

Aedes Dronc*: a novel ecdysone-inducible caspase in the yellow fever mosquito, *Aedes aegypti

D. M. Cooper*, E. P. Thi*‡, C. M. Chamberlain*,
F. Pio† and C. Lowenberger*

*Department of Biological Sciences and †Molecular Biology and Biochemistry, Simon Fraser University, Burnaby B.C. Canada

Abstract

Caspases are cysteinyl-aspartate-specific proteases known for their role in apoptosis. Here, we describe the characterization of *Aedes Dronc*, a novel caspase in the yellow fever mosquito, *Aedes aegypti*. *Aedes Dronc* is predicted to contain an N-terminal caspase recruitment domain and is a homologue of *Drosophila Dronc* and human caspase-9. An increase in transcripts and caspase activity coincides with developmental changes in the mosquito, suggesting that *Aedes Dronc* plays a role in developmental apoptosis. Exposure of third instar larvae to ecdysone resulted in a significant increase in both transcript levels and caspase activity. We present here a functional characterization of the first caspase recruitment domain-containing caspase in mosquitoes, and will initiate studies on the role of apoptosis in the innate immune response of vectors.

Keywords: apoptosis, caspase, *Aedes aegypti*, ecdysone, development.

Introduction

Programmed cell death, in particular apoptosis, is an essential component of metazoan development, and contributes towards tissue homeostasis and immunity (Hengartner, 2000; Kaufmann & Hengartner, 2001; Lawen, 2003). The regulation and control of apoptotic cell death is a highly conserved process culminating in the activation

of a family of cysteine-specific proteases called caspases. Caspases are expressed as zymogens and contain an N-terminal prodomain, and a C14 peptidase domain containing a large (17–20 kDa) and small subunit (10–12 kDa) (Teodoro & Branton, 1997; Raff, 1998; Thornberry & Lazebnik, 1998; Sehra & Dent, 2006). Caspases are activated by proteolytic cleavage at sites located between these domains that removes the prodomain and releases the large and small subunits. Apoptotic caspases are divided into two classes (Sehra & Dent, 2006). The initiator caspases (human caspases-8, -9, -10) are characterized by the presence of long N-terminal prodomains that contain protein–protein interaction motifs such as caspase recruitment domains (CARDs) or a pair of death effector domains (DEDs) (Nicholson, 1999; Ho & Hawkins, 2005). These protein interaction motifs are used to recruit initiator caspases to multi-protein complexes that result in their auto-activation. Once activated, the initiator caspases cleave and activate the second class of caspases, the effector caspases (human caspases-3, -6, -7), characterized by the presence of short N-terminal prodomains. Effector caspases execute the cell death process by cleaving a large number of cellular proteins that bring about the dismantling of the cell.

The *Drosophila* genome contains seven caspase genes (Kumar & Doumanis, 2000; Richardson & Kumar, 2002). Of these, *Dronc*, *Dredd* and *Strica* are predicted to be initiator caspases, whereas the remaining four, *Drice*, *Dcp-1*, *Damm* and *Decay* are predicted to be effector caspases. *Dronc* and *Dredd* carry N-terminal CARD and DED domains respectively, while *Strica* contains a large N-terminal prodomain but lacks any known death domain motifs (Chen *et al.*, 1998; Dorstyn *et al.*, 1999; Adrain & Martin, 2001). *Dronc* is the only known *Drosophila* caspase that carries a CARD motif in its prodomain and is functionally most similar to human caspase-9.

The function of individual caspases in different cell death pathways is just beginning to be understood. *Drosophila* caspases function in both immune signalling pathways and in insect development. Insect metamorphosis is a complex, strictly regulated process/progression of developmental events that is triggered by a number of signals, including

Received 12 January 2007; accepted after revision 12 June 2007; first published online 28 August 2007. Correspondence: Dawn M. Cooper, Department of Biological Sciences, Simon Fraser University, Burnaby B.C. Canada, V5A 1S6. Tel. 1-778-782-4391; fax: 1-778-782-3496; e-mail: dmcooper@sfu.ca

‡Present address: Division of Infectious Disease, Faculty of Medicine, University of British Columbia, 452D, HPE, VGH, Vancouver B.C. Canada, V5Z 3J5

the steroid hormone ecdysone. During metamorphosis some tissues, such as the salivary glands and gut, are completely histolysed, while other predetermined imaginal tissues differentiate and grow into organs and external structures (Lee *et al.*, 2002). A number of *Drosophila* apoptotic proteins are involved in developmental cell death, including Dronc, Reaper and Hid. *Drosophila* Dronc is ubiquitously expressed throughout development and is a known target of ecdysone; an elevation in *dronc* transcripts correlates with ecdysone peaks and the degradation of the larval midgut and salivary gland tissues (Cakouros *et al.*, 2004). All *dronc* mutant alleles are early pupal lethal when homozygous, demonstrating the essential role for *dronc* during development (Daish *et al.*, 2004; Chew *et al.*, 2004; Xu *et al.*, 2005; Waldhuber *et al.*, 2005; Kondo *et al.*, 2006).

Little is known about apoptosis in mosquitoes, although apoptotic-like cell death has been reported previously in the midgut cells and ovarian follicles of *Plasmodium*-infected *Anopheles gambiae* (Zieler & Dvorak, 2000; Al-Olayan *et al.*, 2002; Hurd *et al.*, 2006). While this activity was shown to be caspase-like, the caspases involved in this process were not characterized (Al-Olayan *et al.*, 2002; Hurd *et al.*, 2006). We present here the identification and characterization of *Aedes* Dronc, the first CARD domain-carrying caspase in the yellow fever mosquito, *Aedes aegypti*. *Aedes* Dronc is a homologue of *Drosophila* Dronc and human caspases-2 and -9. *Aedes* Dronc contains the well conserved C14 peptidase subunit with a unique sequence, SIRCG, surrounding the catalytic Cys. Recombinant *Aedes* Dronc exhibits caspase activity on substrates specific for human caspases-9 and -2. *Aedes* Dronc also contains a putative N-terminal CARD domain, suggesting that an intrinsic apoptotic pathway exists in mosquitoes. As is the case in *Drosophila*, *Aedes* Dronc appears to be involved principally in insect development with the highest levels of transcripts and caspase activity detected in late instar larvae and early and late pupae, which corresponds to tissue reorganization and pulses of the steroid hormone ecdysone.

Results

Identification of *Aedes* Dronc

We performed a TBLASTN search of the TIGR *Aedes* genomic database using protein sequence queries of both the C14 peptidase subunits and the prodomains of *Drosophila* and mammalian caspases and identified a draft sequence that shared 36% identity with *Drosophila* Dronc. Through 5'-3' RACE PCR we obtained a cDNA with an open reading frame that encodes a novel 449 residue caspase named *Aedes* Dronc. *Aedes* Dronc shares 99% identity with a putative uncharacterized protein present in the NCBI database (GENBANK accession: gi:78216591). Phylogenetic analysis clusters *Aedes* Dronc with *Drosophila*

Dronc and human caspases-1, -2 and -9 (Fig. 1). Comparisons with the conserved domain database indicate that *Aedes* Dronc contains a C-terminal C14 peptidase subunit [251 amino acids (aa)] and an N-terminal prodomain 198 aa in length. Multiple sequence alignments with mammalian and invertebrate caspases indicate that *Aedes* Dronc contains all the essential residues required for catalysis (His-237, Gly-238 and Cys-285) and the residues involved in coordinating the P1 aspartate (Arg-179, Gln-283, Arg-341, Ser-343), using caspase-1 nomenclature (Fig. 2A) (Earnshaw *et al.*, 1999). The C-terminus of *Aedes* Dronc shares significant sequence identity with the C14 peptidase subunits of all mammalian and insect caspases, with the highest similarity with *Drosophila* Dronc (36% identity, 55% similarity). *Aedes* Dronc shares 29% identity (49% similarity) with the C14 peptidase subunit of human caspase-2, 29% identity (49% similarity) with human caspase-9 (Fig. 2A). The sequence encompassing the catalytic Cys in *Aedes* Dronc is unique and contains SICRG.

Although caspase prodomains tend to be much more divergent than the catalytic segments, we identified limited sequence similarities among the prodomains of *Aedes* Dronc, *Drosophila* Dronc, human caspase-2 and human caspase-9. The prodomain of *Aedes* Dronc shares the greatest identity with the prodomain of *Drosophila* Dronc (31% identity; 54% similarity), and limited sequence identity with the prodomains of human caspase-2 (10% identity; 20% similarity) and human caspase-9 (10% identity; 19% similarity). We used a fold recognition program (BioInfoBank Metaserver; <http://meta.bioinfo.pl>) to predict secondary structure in the prodomain of *Aedes* Dronc. The program predicts one six- α -helix bundle in the prodomain of *Aedes* Dronc, nearly identical to that found in *Drosophila* Dronc and similar to those found in human caspases-2 and -9 (Fig. 2B).

Expression during development

Quantitative real-time PCR (qPCR) detected *Aedes dronc* transcripts in all developmental stages. Transcript levels in third instar larvae were 26-fold higher than levels in the early instar stages. Transcript levels in fourth instar larvae were 12-fold higher than those in the third instar larvae, while transcript levels in callow and black pupae were 126 and 53-fold higher, respectively, than third instar larvae (Fig. 3A).

Tissue-specific expression

Aedes dronc transcripts were found in differing levels in all individual adult tissues screened in this study. Transcript levels were highest in the fat body and gut tissues; six- to eight-fold higher than the levels found in salivary glands and approximately two-fold higher than ovary tissues (Fig. 3B).

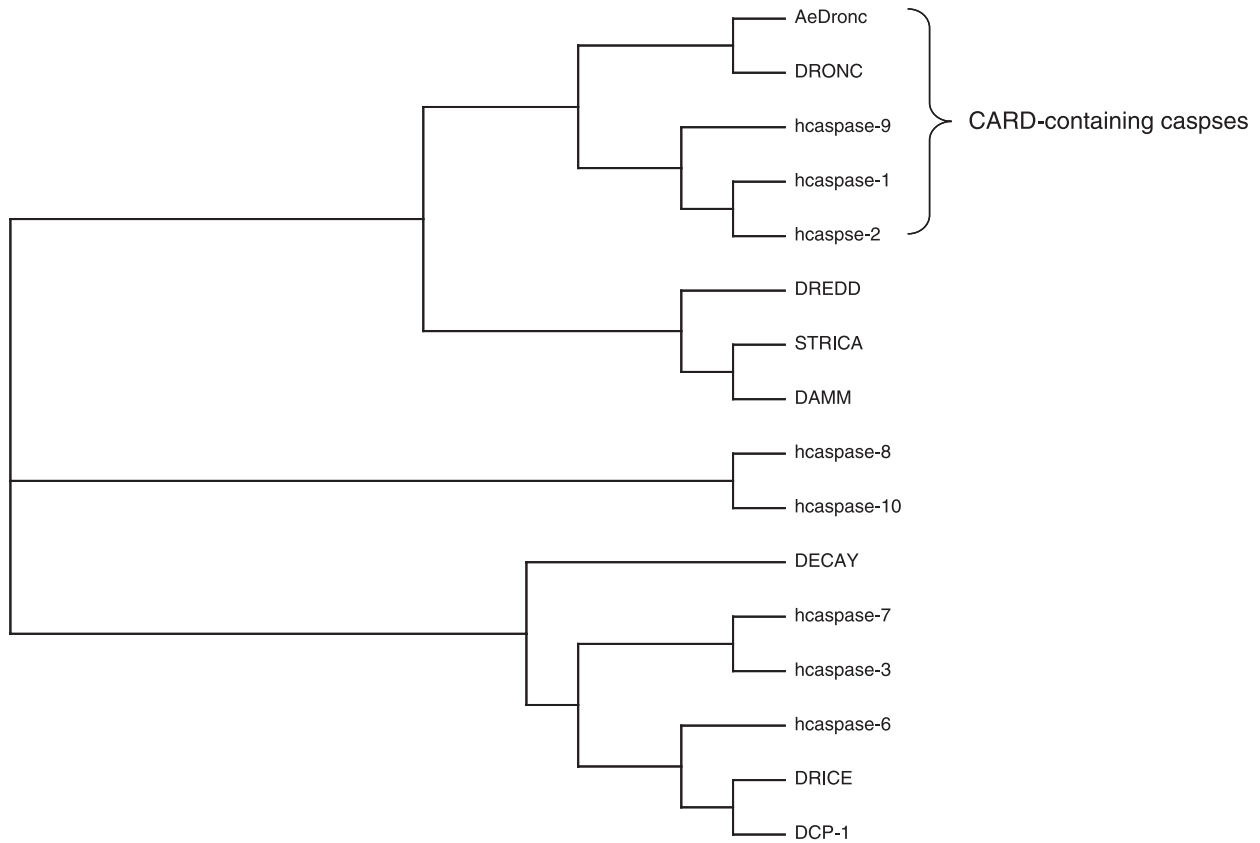


Figure 1. Phylogenetic analysis of *Aedes Dronc* with *Drosophila* (capitalized) and human (denoted with an h) caspases. Multiple sequence alignments were performed using Clustal X and tree was produced using the neighbor-joining method and TreeView.

Enzymatic activity and substrate specificity of recombinant *Aedes Dronc*

We expressed and incubated the C14 peptidase subunit of *Aedes Dronc* with a variety of caspase-specific colorimetric tetrapeptide substrates to analyse substrate preference. *Aedes Dronc* displayed significant cleavage on VDVAD-pNA (optimal for caspase-2) and LEHD-pNA (optimal for caspase-9) (Fig. 4A). Minimal activity was also detected on AEVD-pNA (optimal for caspase-10) and WEHD-pNA (optimal for caspase-5). The activity of *Aedes Dronc* on VDVAD-pNA was suppressed by all concentrations of the general caspase family inhibitor z-VAD-fmk (Fig. 4B).

Caspase activity during development

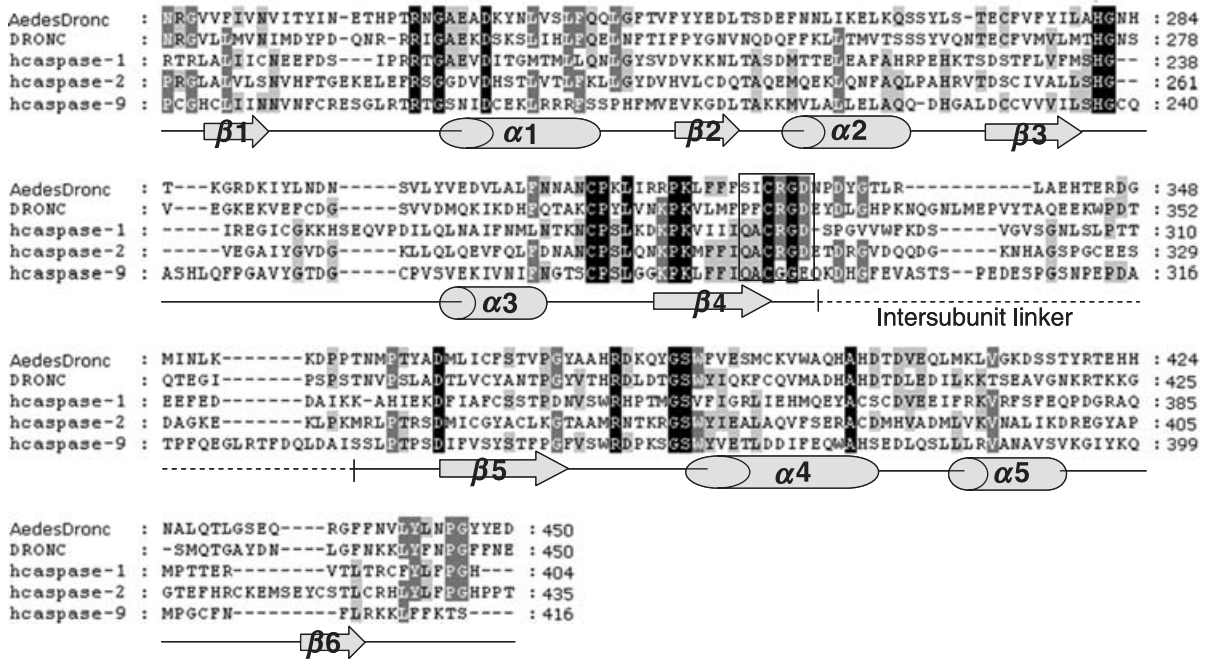
We assessed caspase activity during different developmental stages of *Ae. aegypti* by mixing total cell protein extracted from early instar, late instar, callow and black pupae with the caspase-3 substrate DEVD-pNA and the caspase-9 substrate LEHD-pNA. Although recombinant *Aedes Dronc* was shown to prefer VDVAD-pNA, it was not used in this developmental assay because previous work in our lab indicates that other mosquito caspases also use VDVAD-pNA (data not shown). Consistent with our

expression data, cell extracts prepared from late instar larvae, early (callow) and late (black) pupae showed the highest activity on the caspase-9 substrate, suggesting that the increase in *Aedes dronc* transcripts correlates with active *Aedes Dronc* protein. In addition, subsequent caspase-3-like activity was detected in all developmental stages, suggesting involvement of downstream effector caspases (Fig. 5). Caspase activity in the developmental assays was greatly reduced by the addition of 2 μ M z-VAD-fmk, confirming that the activity we were measuring was caspase specific.

Ecdysone treatment

We examined transcript levels of *Aedes dronc* in third instar larvae after a 24 or 48 h exposure to ecdysone. We detected a six-fold increase at 24 h and a 16-fold increase in *Aedes dronc* transcripts after 48 h of exposure (Fig. 6A). An analysis of total cell protein from larvae treated with ecdysone showed an increase in both *Aedes Dronc* specific activity (LEHD-pNA) and caspase-3-like activity (DEVD-pNA). LEHD-pNA activity increased to 24 h and remained relatively constant to 48 h, whereas the activity on DEVD-pNA steadily increased to 48 h (Fig. 6B).

(A)



(B)

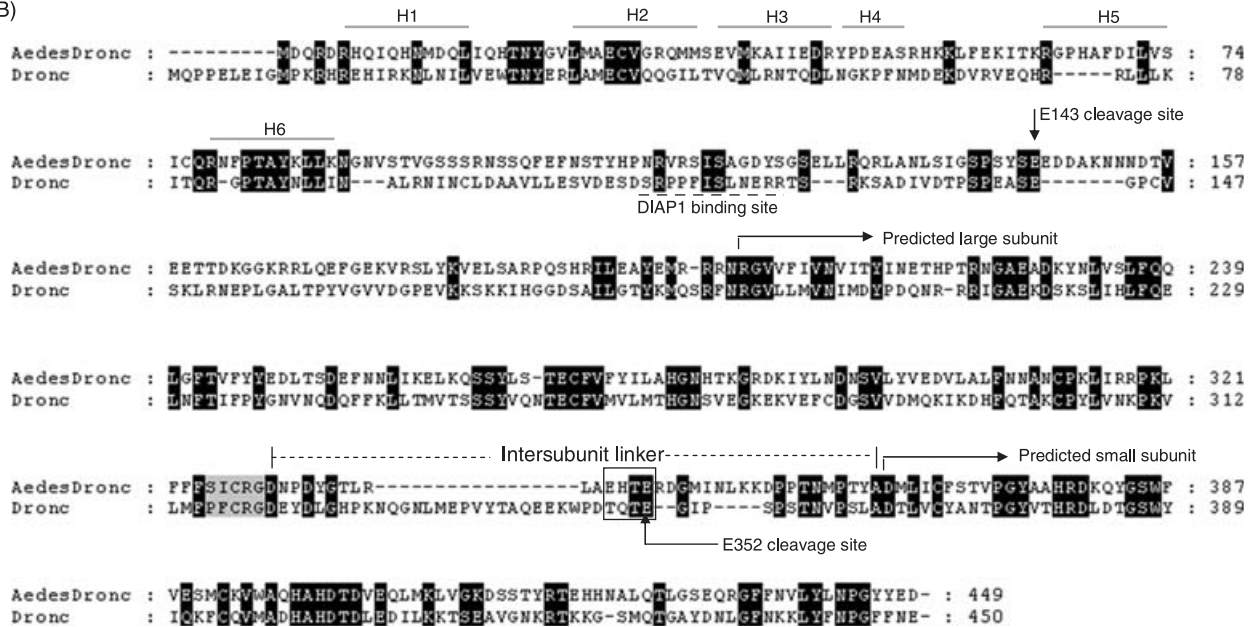


Figure 2. (A) Multiple sequence alignment of the partial enzymatic domains of *Aedes Dronc* with human and *Drosophila* caspases. Residues shared by all proteins are highlighted in black, those shared by four proteins are darkly shaded and those shared by three proteins are lightly shaded. Spatially aligned α -helices and β -sheets are indicated. The pentapeptide sequence surrounding the catalytic cysteine is boxed. (B) An alignment of the deduced amino acid sequence of *Aedes Dronc* and *Drosophila Dronc*. The positions of the α -helices (H1–H6) in the CARD domains were predicted using the BioInfoBank Metaserver (<http://meta.bioinfo.pl/>). The known 12-residue sequence required for DIAP1 binding in *Drosophila Dronc* is underlined (dashed line). The catalytic site containing the unique pentapeptide sequence is shaded in grey. The conserved E143 cleavage site (*Drosophila Dronc* nomenclature) is denoted by a vertical arrow and the tetrapeptide sequence containing the conserved E352 cleavage site in the intersubunit linker that separates the large and small catalytic subunits is boxed. Alignments were constructed using CLUSTALX.

Discussion

Caspases are a family of cysteine proteases responsible for the concerted and coordinated action of apoptosis.

Caspases are expressed as pro-enzymes composed of three domains: an N-terminal prodomain and a large and small catalytic subunit. Activation requires proteolytic cleavage among these domains and the formation of heterodimers

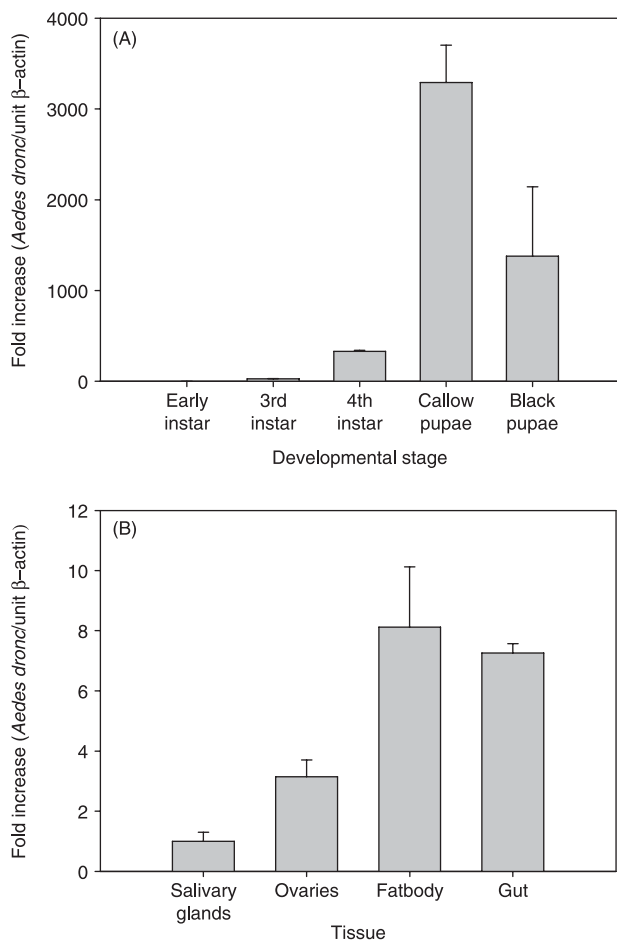


Figure 3. Relative expression of *Aedes dronc* in (A) different developmental stages and (B) individual adult tissues using real-time quantitative PCR. Expression levels from early instar larvae (A) and salivary gland tissues (B) were arbitrarily set to 1. The vertical axis represents the fold increase in transcript number in different developmental stages or different adult tissues relative to the assigned control. *Aedes dronc* values were normalized using the housekeeping gene β -actin. Error bars represent SD.

by the large and small subunits (Thornberry & Lazebnik, 1998). These inter-domain cleavage sites are themselves caspase consensus sites, indicating that caspases function as part of a proteolytic cascade (Cohen, 1997) that involves the auto- and trans-activation of apical caspases which then cleave and activate downstream effector caspases (Nicholson, 1999). Apoptotic caspases are classified as initiators which contain long prodomains, and effectors which contain short prodomains.

The large prodomains of initiator caspases contain CARD (human caspases-1, -2, -4, -5, -9) and DED (human caspase-8 and -10) domains that are used to recruit the initiator caspases to multi-protein complexes for auto-activation (Nicholson, 1999; Ho & Hawkins, 2005). Four initiator caspases have been identified in insects: *Drosophila Dronc*, which carries a CARD domain; *Drosophila Dredd*, which carries two DEDs; *Drosophila Strica*, which carries a

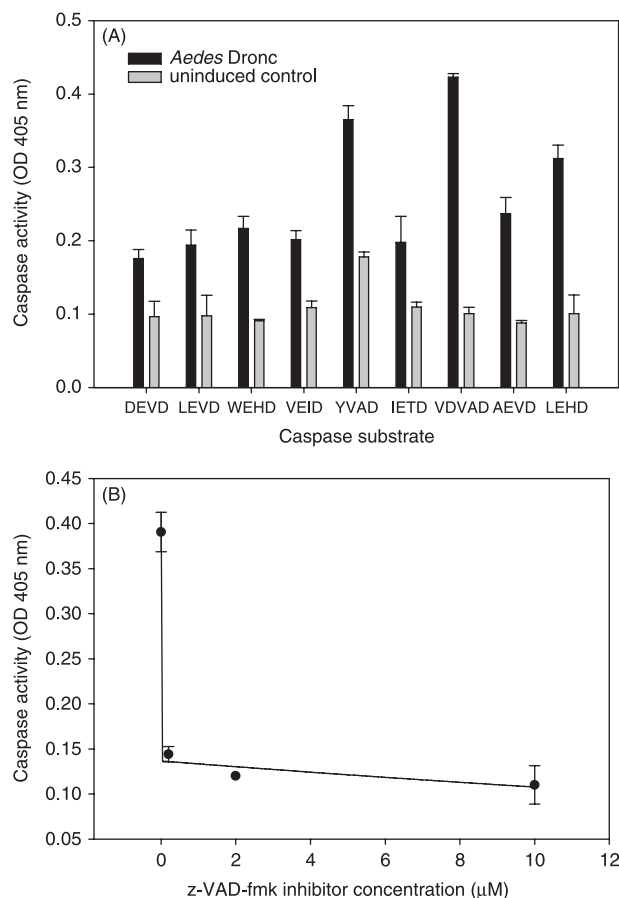


Figure 4. Substrate specificity of *Aedes Dronc*. (A) Activity of recombinant *Aedes Dronc* on caspase-specific colorimetric peptide substrates. Equivalent amounts of uninduced *Escherichia coli* containing *Aedes Dronc* lysates were used as controls. (B) *E. coli* lysates containing the recombinant C14 subunit of *Aedes Dronc* were treated with varying amounts of the general caspase inhibitor, z-VAD fmk. z-VAD fmk inhibited caspase activity, as measured by the ability of recombinant *Aedes Dronc* to use VDAVD-pNA, at concentrations as low as 0.2 μ M. Error bars represent SD.

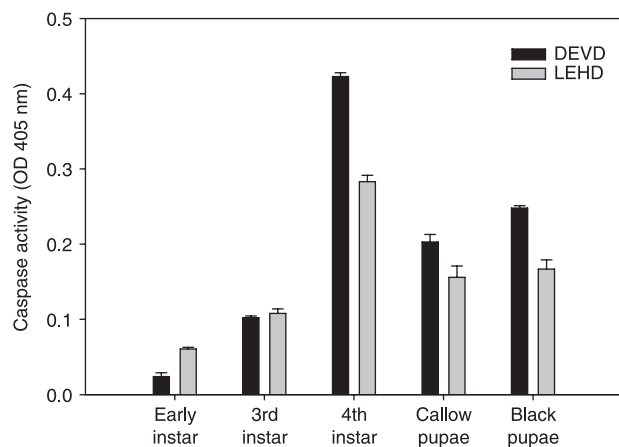


Figure 5. Caspase activity during development. Total cell protein from whole mosquitoes was combined with 200 μ M LEHD-pNA or DEVD-pNA and incubated at 37 $^{\circ}$ C for 3 h. LEHD-pNA was used to measure specific *Aedes Dronc* activity and DEVD-pNA was used to measure downstream caspase-3 like activity. Error bars represent SD.

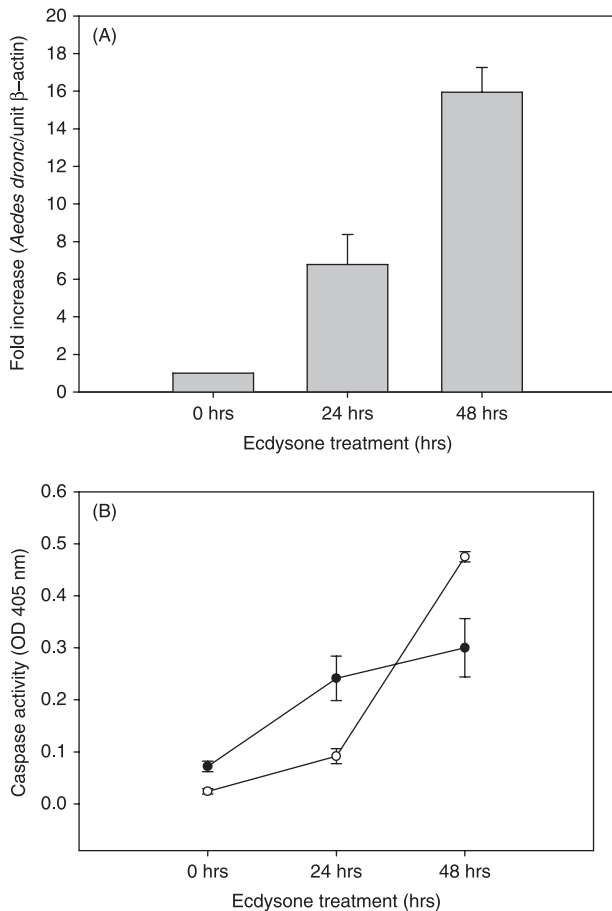


Figure 6. Ecdysone induces *Aedes dronc* expression and caspase activity in third instar larvae. Thirty early third instar larvae were treated with 30 μ M ecdysone for 24 or 48 h. (A) Relative expression of *Aedes dronc* during ecdysone treatments as measured by real-time quantitative PCR. The expression values from larvae collected at 0 h were arbitrarily set to 1 and the vertical axis represents fold increase in expression after ecdysone exposure. (B) Caspase activity in third instar larvae treated with 30 μ M ecdysone. 100 μ g total cell protein was assayed for caspase activity using LEHD-pNA (●) and DEVD-pNA (○). Error bars represent SD. Data represent duplicate treatments.

serine/threonine rich region; and *Aedes Dredd* which carries two DEDs (Chen *et al.*, 1998; Dorstyn *et al.*, 1999; Kumar & Doumanis, 2000; Adrain & Martin, 2001; Cooper *et al.*, 2007). CARD and DED are homotypic domains required for the transmission and regulation of signals from death receptors to apoptotic effector molecules, such as the caspases (Tibbetts *et al.*, 2003). In general, these homotypic interaction domains share a low degree of sequence similarity but share a common three-dimensional fold, classified as the death domain-fold in SCOP (Structural Classification of Proteins) (Murzin *et al.*, 1995). Even within the death domain family, the sequences can be so divergent that statistically significant sequence similarities cannot be detected by conventional sequence comparison. The death domain-fold comprises an antiparallel six-helix bundle. By sequence comparison, the prodomain of *Aedes*

Dronc shares 31% sequence identity with the prodomain of *Drosophila Dronc*, 10% identity with the prodomain of human caspase-2 and 10% identity with the prodomain of caspase-9. Despite the lack of obvious sequence similarity, the prodomain of *Aedes Dronc* is predicted to contain one six- α -helix bundle, nearly identical to that seen in *Drosophila Dronc* (Fig. 2B), indicating that *Aedes Dronc* is a CARD-carrying caspase.

In addition to N-terminal prodomains, caspases contain a conserved C14 peptidase domain, comprising a large (17–20 kDa) and small (10–12 kDa) subunit. The catalytic domain of *Aedes Dronc* shares a high degree of sequence similarity with the catalytic domains of many known caspases, with the highest similarity found with *Drosophila Dronc*. *Aedes Dronc* contains all the residues involved in caspase catalysis; however, the sequence encompassing the catalytic Cys residue in *Aedes Dronc* is SICRG, different from the QAC(R/Q/G)(G/E) found in most known caspases. *Drosophila Dronc* also has a unique catalytic sequence, PFCRG, surrounding the catalytic Cys (Fig. 2A), which accounts for its unique specificity (Dorstyn *et al.*, 1999). *Aedes Dronc* showed significant activity on VDVA-pNA and LEHD-pNA and minimal activity on WEHD-pNA and AEVD-pNA (Fig. 4A). LEHD-pNA is a substrate preferred by human caspase-9 while VDVA-pNA is the optimal substrate for caspase-2. This differs from the substrate specificity observed with *Drosophila Dronc*, which also shows activity on VEID, IETD and VDVA substrates, and minimal activity on DEVD-containing substrates (Dorstyn *et al.*, 1999; Hawkins *et al.*, 2000).

In general, caspases show a high degree of specificity, and cleave after an aspartic acid (Talanian *et al.*, 1997). *Drosophila Dronc* is unique among caspases because it will cleave after an aspartate residue (similar to other caspases) but also will cleave after glutamate residues (Hawkins *et al.*, 2000; Muro *et al.*, 2004; Hay & Guo, 2006). *Dronc* activation involves cleavage at E352, triggering the transition from an inactive monomer to a catalytically active dimer (Hawkins *et al.*, 2000; Muro *et al.*, 2004; Hay & Guo, 2006). Following cleavage at E352, *Dronc* undergoes autocleavage at E143 (Muro *et al.*, 2004; Yan *et al.*, 2006), releasing the CARD domain from the active catalytic dimer.

Sequence analysis indicates that both E143 and E352 are conserved in *Aedes Dronc* (Fig. 2B), suggesting that *Aedes Dronc* activation proceeds through a similar mechanism. The specific activation and ability of *Aedes Dronc* to cleave after a glutamate will be the subject of future studies. Cleavage after E143 in *Drosophila Dronc* removes the DIAP1 binding site. DIAP1 belongs to the IAP (Inhibitors of Apoptosis Proteins) family of proteins which can inhibit caspase function by binding to the active caspase site, by promoting degradation of active caspases or by sequestering the caspases away from their substrates (Tenev *et al.*, 2005). DIAP1 contains a BIR domain that binds to a 12-residue

sequence in Dronc (114-SRPPFISLNFERR-125) located between the CARD domain and the large catalytic subunit (Chai *et al.*, 2003). The C-terminal RING finger domain of DIAP1 then targets Dronc for ubiquitination (Wilson *et al.*, 2002; Chai *et al.*, 2003). There is conservation, albeit limited, of the 12-residue DIAP1 binding site in *Aedes* Dronc, suggesting that *Aedes* Dronc activity may also be regulated by a DIAP1 homologue in mosquitoes (Fig. 2B). Two IAP molecules, AtIAP and AalIAP1, have been identified in mosquitoes (Blitvich *et al.*, 2002; Li *et al.*, 2007). AalIAP1 appears to be the mosquito homologue of DIAP1 (Li *et al.*, 2007) and investigation of the ability of AalIAP1 to interact with and inhibit *Aedes* Dronc activity is currently underway.

Aedes dronc transcripts were found in all adult tissues screened in this study, with the highest levels found in the fat body, a tissue with high cell turnover rates and the primary immune response tissue in insects (Fig. 3B). *Aedes dronc* was ubiquitously expressed during mosquito development, with the highest levels of transcript found in the pupal stages (Fig. 3A). In insects, the steroid hormone ecdysone regulates cell death and differentiation during development. An ecdysone pulse toward the end of third larval instar development in *Drosophila* induces imaginal disc evagination, a dramatic change in salivary gland gene transcription and larval midgut cell death (Baehrecke, 2000; Jiang *et al.*, 2000; Lee *et al.*, 2002). A second pulse of ecdysone in the early pupal stage then triggers salivary gland cell death. Studies have shown that *dronc* and its effector molecules, *rpr* and *hid*, are barely detectable in early instar larvae but are transcriptionally upregulated in response to the pulses of ecdysone in late third instar larvae prior to developmental apoptosis (Jiang *et al.*, 2000; Daish *et al.*, 2003; Cakouros *et al.*, 2004). Metamorphosis in mosquitoes is similarly triggered by pulses of ecdysone. In the presence of juvenile hormone, ecdysone triggers larval-larval moults (Margam *et al.*, 2006). During the late fourth instar, a small peak of ecdysone, in the absence of juvenile hormone, is responsible for larval commitment to pupal development. A second peak of ecdysone triggers apolysis, followed by formation of the pupal cuticle. In the pupal stage, a large peak of ecdysone triggers apolysis and the initiation of adult tissue formation (Clements, 1992; Margam *et al.*, 2006). The increase in *Aedes dronc* transcripts observed in the fourth instar larvae and the early (callow) and late (black) pupae stages is correlated with both LEHD-pNA activity, which suggests *Aedes* Dronc activity, and with DEVD-pNA which suggests downstream effector caspase activity. Although we do not have a complete substrate usage profile for all of the caspases that exist in mosquitoes, the profile determined for *Aedes* Dronc, in conjunction with the strong upregulation of *Aedes dronc* transcripts, suggests that *Aedes dronc* may also be developmentally regulated and involved in the apoptotic destruction of larval tissues during metamorphosis (Fig. 5).

An increase was detected in *dronc* transcripts in the midgut tissues of fourth instar *Aedes aegypti* larvae but not in the gut tissues of the early pupal stage (Wu *et al.*, 2006). We detected an increase in *dronc* transcripts in the whole bodies of both fourth instar larvae and pupal stages. The additional peak of *Aedes dronc* transcripts in the pupal stages may represent the apoptotic destruction of other tissues such as the salivary glands (Daish *et al.*, 2003; Cakouros *et al.*, 2004). To investigate whether ecdysone induces the expression of *Aedes dronc* in mosquitoes, we examined the levels of *Aedes dronc* transcripts in third instar larvae following treatment with ecdysone. Third instar larvae were chosen because they normally only show low levels of both *dronc* transcripts and caspase activity. We detected a 16-fold increase in *dronc* transcripts and a large increase in both LEHD-pNA and DEVD-pNA caspase activity in larvae 48 h after ecdysone treatment. The activity on LEHD-pNA increased at 24 h and remained relatively constant to 48 h, whereas the activity on DEVD-pNA steadily increased to 48 h. This pattern of activation suggests that *Aedes* Dronc is an initiator caspase responsible for the activation of downstream effector caspases. These data, together with recent studies, suggest that many caspases, including *Aedes* Dronc, mediate tissue and stage-specific apoptosis during insect development (Mills *et al.*, 2005).

Few studies have addressed the role of apoptosis in insects that transmit human parasites. The identification of *Aedes* Dronc as a CARD-containing caspase, and as a homologue of *Drosophila* Dronc, suggests that a Dronc/caspase-9 apoptotic pathway may also exist in mosquitoes. In addition to development, apoptosis is a known mediator of the immune response (Schaumburg *et al.*, 2006). Apoptotic-like activity has been associated with *Plasmodium* infection in *Anopheles gambiae*, suggesting a role for apoptosis in the mosquito immune response (Zieler & Dvorak, 2000; Hopwood *et al.*, 2001; Al-Olayan *et al.*, 2002; Hurd *et al.*, 2006). The identification of a caspase central to a *Drosophila* Dronc/caspase-9 pathway will provide a stepping stone to characterizing the role that apoptosis and caspases play in mosquito immunity. The role of *Aedes* Dronc in the immune response of *Aedes aegypti* will be the subject of further study. Information on the effector molecules that regulate the apoptotic response, such as caspases, will provide insight into the mechanisms that insect vectors use to regulate intracellular infections and provide a means to examine the evolution of apoptotic pathways as they relate to development and immunity.

Experimental procedures

Insect maintenance, cDNA generation

Aedes aegypti (Liverpool strain) mosquitoes were reared as described previously (Lowenberger *et al.*, 1999). Total RNA was

extracted from different developmental stages (larvae, callow pupae, black pupae and whole adult mosquitoes) or from individual tissues (salivary glands, ovaries, midguts and fat body tissues) as described previously (Cooper *et al.*, 2007). RNA concentration was determined using a biophotometer (Eppendorf, Hamburg, Germany) and reverse transcription was performed using 3 µg of total RNA. All reverse transcription reactions were performed as described previously (Lowenberger *et al.*, 1999), with the modification of using a 40 °C incubation temperature for 60 min. All cDNAs were screened with primers known to span an intron to verify that samples contained no genomic DNA contamination.

Identification and sequencing *Aedes Dronc*

Partial sequences of *Aedes Dronc* were identified in a series of TBLASTN searches of the TIGR *Aedes* Gene Indices (<http://www.tigr.org/tdb/tgi/>) as an expressed sequence tag (EST) displaying significant homology to the C14 peptidase subunit of *Drosophila* and mammalian caspases. Primers designed against the EST sequences were used to obtain partial cDNA segments from our whole body and tissue-specific cDNAs. 5'–3' RACE PCR (Marathon cDNA Amplification kit; Clontech, Mountain View, CA, USA) was used to obtain the full length cDNA. Purified PCR products were cloned into pGEM T-Easy (Promega, Madison, WI, USA) and sequenced using Big Dye v3.1 chemistry (ABI, Foster City, CA, USA). Sequence information was used to design primers for qPCR studies.

Tissue-specific and developmental comparisons

For an estimation of tissue-specific expression and mRNA concentration we used qPCR. All qPCR reactions were performed with a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using the Platinum SYBR Green Supermix-UDG (Invitrogen, Carlsbad, CA, USA). We used 2.5 µl cDNA with 12.5 µl of Platinum SYBR Green Supermix, 1 µl (10 µM) sense primer (5'-CGAAGCGGACAAG-GACAAC-3'), 1 µl (10 µM) antisense primer (5'-GCGACAGAT-GGAGAAAAGAAC-3') in 25 µl reactions under the following conditions: 50 °C for 2 min, 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 61.5 °C for 15 s, 72 °C for 30 s. Quantity values were generated using the 2(-ΔΔCT) method as described previously (Livak & Schmittgen, 2001). The expression of *Aedes dronc* was compared per unit of *β-actin*, a normalizing gene that had stable expression levels in all conditions tested. All data represent triplicate runs of independently generated cDNAs.

Expression of recombinant *Aedes Dronc*, caspase assays, and inhibition assays

Recombinant *Aedes Dronc* was generated by transforming *Escherichia coli* BL21 (E3) cells with a truncated form of *Aedes Dronc* lacking the putative prodomain (residues 1–198) (Fig. 1A). The C14 subunit was amplified by PCR and cloned into pET-46 EK/LIC (Novagen, Madison, WI, USA). pET-*Aedes Dronc* LB-Amp⁵⁰ starter cultures were grown for 3 h, subcultured into fresh medium and grown at 37 °C to an Optical Density (OD) of 0.5. Cultures were induced with 2 mM IPTG and grown for 16 h at 22 °C. Cells were pelleted and lysed using Easy-Lyse Bacterial Protein Extraction Solution (Epicentre, Madison, WI, USA) following the manufacturer's instructions. Cleared *E. coli* lysates from cells expressing *Aedes Dronc* were assayed for protein concentration using the Bradford Assay (BioRad, Hercules, CA, USA), and 100 µg of total

protein from each lysate was incubated with 200 µM chromophore p-nitroaniline-labelled substrates for 3 h at 37 °C according to the manufacturer's recommendations (BioVision Inc., Mountain View, CA, USA). The release of –pNA was monitored by a spectrophotometer at 405 nm. Activity was considered biologically significant when absorbance was two-fold higher than the uninduced controls (Biovision Inc.). We used the general caspase inhibitor, z-VAD-fmk (Biovision Inc.), to inhibit caspase activity by interfering with the processing of the substrate VDVA-pNA. For each assay, a given amount of z-VAD-fmk was mixed with 100 µg of *Aedes Dronc* lysate (total protein) and allowed to incubate for 1 min. 200 µM VDVA-pna was added and each reaction was allowed to incubate for 3 h at 37 °C. The data represent three replicates, measured in duplicate.

Caspase activity in developmental stages

To determine if changes in gene expression were correlated with caspase activity, we measured caspase-9 and caspase-3 activity in cell extracts prepared from early instar, late instar and callow and black pupae. Larvae or pupae were ground in 1× Phosphate Buffered Saline (PBS) and frozen at –80 °C. Cells were fractured with four freeze–thaw cycles (–80 °C for 5 min and fast thawed for 30 s at 42 °C). Insect carcasses were removed by centrifugation at 10 000 *g* for 1.5 min. Cells were lysed with 200 µl cell lysis solution (Biovision Inc.). Phenylthiourea (15 mM final concentration) was added to each cell extract to prevent melanization. Total cell protein was measured as described above and 100 µg total cell protein from each cell lysate was incubated with 200 µM DEVD-pNA or LEHD-pNA for 3 h at 37 °C according to the manufacturers' recommendations. The release of –pNA was monitored by a spectrophotometer at 405 nm. To ensure the activity we were detecting in these assays was the result of caspase specific activity, we added 2 µM z-VAD-fmk to 100 µg total cell protein as described above.

Ecdysone treatments

Early third instar larvae were rinsed with fresh ddH₂O and maintained individually for 3 h in tissue culture wells containing 100 µl of water and 30 µM 20-hydroxyecdysone (ecdysone) (Sigma, Oakville, ON, Canada) for 24 and 48 h at 22–25 °C. Water volumes were increased to 1 ml/well and finely ground TetraMin® (Montreal, QC, Canada) fish flakes were added as a food source 3 h after initial ecdysone exposure. At specific time points, whole larvae were placed directly in Tri-Reagent and RNA extraction, cDNA synthesis and qPCR were performed as described above. Larvae treated with water served as the control. Caspase activity at 0, 24 and 48 h after ecdysone treatment was assessed using 200 µM LEHD-pNA or DEVD-pNA and was conducted as described above.

Acknowledgements

We thank K. Foster and C. Perez for raising mosquitoes, K. Dalal for help with protein expression and R. Plunkett and R. Ursic-Bedoya for helpful comments on the manuscript. This work was supported in part by a MSFHR trainee grant to DC, NSERC (611306) to FP, and NSERC (261940), CIHR (69558), the Canada Research Chair program and a MSFHR scholar award to CL.

References

- Adrain, C. and Martin, S.J. (2001) Search for *Drosophila* caspases bears fruit: STRICA enters the fray. *Cell Death Differ* **8**: 319–323.
- Al-Olayan, E.M., Williams, G.T. and Hurd, H. (2002) Apoptosis in the malaria protozoan, *Plasmodium berghei*: a possible mechanism for limiting intensity of infection in the mosquito. *Int J Parasitol* **32**: 1133–1143.
- Baehrecke, E.H. (2000) Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ* **7**: 1057–1062.
- Blitvich, B.J., Blair, C.D., Kempf, B.J., Hughes, M.T., Black, W.C., Mackie, R.S., et al. (2002) Developmental- and tissue-specific expression of an inhibitor of apoptosis protein 1 homologue from *Aedes triseriatus* mosquitoes. *Insect Mol Biol* **11**: 431–442.
- Cakouros, D., Daish, T.J. and Kumar, S. (2004) Ecdysone receptor directly binds the promoter of the *Drosophila* caspase *dronc*, regulating its expression in specific tissues. *J Cell Biol* **165**: 631–640.
- Chai, J., Yan, N., Huh, J.R., Wu, J.W., Li, W., Hay, B.A., et al. (2003) Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. *Nat Struct Biol* **10**: 892–898.
- Chen, P., Rodriguez, A., Erskine, R., Thach, T. and Abrams, J.M. (1998) Dredd, a novel effector of the apoptosis activators reaper, grim, and hid in *Drosophila*. *Dev Biol* **201**: 202–216.
- Chew, S.K., Akdemir, F., Chen, P., Lu, W.J., Mills, K., Daish, T., et al. (2004) The apical caspase *dronc* governs programmed and unprogrammed cell death in *Drosophila*. *Dev Cell* **7**: 897–907.
- Clements, A.N. (1992) *The Biology of Mosquitoes*, Chapman and Hall, New York.
- Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J* **326** (Pt 1): 1–16.
- Cooper, D.M., Pio, F., Thi, E.P., Theilmann, D. and Lowenberger, C. (2007) Characterization of *Aedes* Dredd: a novel initiator caspase from the yellow fever mosquito, *Aedes aegypti*. *Insect Biochem Mol Biol* **37**: 559–569.
- Daish, T.J., Cakouros, D. and Kumar, S. (2003) Distinct promoter regions regulate spatial and temporal expression of the *Drosophila* caspase *dronc*. *Cell Death Differ* **10**: 1348–1356.
- Daish, T.J., Mills, K. and Kumar, S. (2004) *Drosophila* caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. *Dev Cell* **7**: 909–915.
- Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H. and Kumar, S. (1999) DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc Natl Acad Sci USA* **96**: 4307–4312.
- Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* **68**: 383–424.
- Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y. and Hay, B.A. (2000) The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J Biol Chem* **275**: 27084–27093.
- Hay, B.A. and Guo, M. (2006) Caspase-dependent cell death in *Drosophila*. *Annu Rev Cell Dev Biol* **22**: 623–650.
- Hengartner, M.O. (2000) The biochemistry of apoptosis. *Nature* **407**: 770–776.
- Ho, P.K. and Hawkins, C.J. (2005) Mammalian initiator apoptotic caspases. *FEBS J* **272**: 5436–5453.
- Hopwood, J.A., Ahmed, A.M., Polwart, A., Williams, G.T. and Hurd, H. (2001) Malaria-induced apoptosis in mosquito ovaries: a mechanism to control vector egg production. *J Exp Biol* **204**: 2773–2780.
- Hurd, H., Grant, K.M. and Arambage, S.C. (2006) Apoptosis-like death as a feature of malaria infection in mosquitoes. *Parasitology* **132** (Suppl 1): S33–47.
- Jiang, C., Lamblin, A.F., Steller, H. and Thummel, C.S. (2000) A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol Cell* **5**: 445–455.
- Kaufmann, S.H. and Hengartner, M.O. (2001) Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* **11**: 526–534.
- Kondo, S., Senoo-Matsuda, N., Hiromi, Y. and Miura, M. (2006) DRONC coordinates cell death and compensatory proliferation. *Mol Cell Biol* **26**: 7258–7268.
- Kumar, S. and Doumanis, J. (2000) The fly caspases. *Cell Death Differ* **7**: 1039–1044.
- Lawen, A. (2003) Apoptosis—an introduction. *Bioessays* **25**: 888–896.
- Lee, C.Y., Cooksey, B.A. and Baehrecke, E.H. (2002) Steroid regulation of midgut cell death during *Drosophila* development. *Dev Biol* **250**: 101–111.
- Li, Q., Li, H., Blitvich, B.J. and Zhang, J. (2007) The *Aedes albopictus* inhibitor of apoptosis 1 gene protects vertebrate cells from bluetongue virus-induced apoptosis. *Insect Mol Biol* **16**: 93–105.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25**: 402–408.
- Lowenberger, C., Charlet, M., Vizioli, J., Kamal, S., Richman, A., Christensen, B.M., et al. (1999) Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the cecropin family from the mosquito vector *Aedes aegypti*. *J Biol Chem* **274**: 20092–20097.
- Margam, V.M., Gelman, D.B. and Palli, S.R. (2006) Ecdysteroid titers and developmental expression of ecdysteroid-regulated genes during metamorphosis of the yellow fever mosquito, *Aedes aegypti* (Diptera: Culicidae). *J Insect Physiol* **52**: 558–568.
- Mills, K., Daish, T. and Kumar, S. (2005) The function of the *Drosophila* caspase DRONC in cell death and development. *Cell Cycle* **4**: 744–746.
- Muro, I., Monser, K. and Clem, R.J. (2004) Mechanism of Dronc activation in *Drosophila* cells. *J Cell Sci* **117**: 5035–5041.
- Murzin, A.G., Brenner, S.E., Hubbard, T. and Chothia, C. (1995) SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* **247**: 536–540.
- Nicholson, D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* **6**: 1028–1042.
- Raff, M. (1998) Cell suicide for beginners. *Nature* **396**: 119–122.
- Richardson, H. and Kumar, S. (2002) Death to flies: *Drosophila* as a model system to study programmed cell death. *J Immunol Methods* **265**: 21–38.
- Schaumburg, F., Hippe, D., Vutova, P. and Luder, C.G. (2006) Pro- and anti-apoptotic activities of protozoan parasites. *Parasitology* **132** (Suppl 1): S69–85.
- Sehra, S. and Dent, A.L. (2006) Caspase function and the immune system. *Crit Rev Immunol* **26**: 133–148.

- Talanian, R.V., Quinlan, C., Trautz, S., Hackett, M.C., Mankovich, J.A., Banach, D., *et al.* (1997) Substrate specificities of caspase family proteases. *J Biol Chem* **272**: 9677–9682.
- Tenev, T., Zachariou, A., Wilson, R., Ditzel, M. and Meier, P. (2005) IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. *Nat Cell Biol* **7**: 70–77.
- Teodoro, J.G. and Branton, P.E. (1997) Regulation of apoptosis by viral gene products. *J Virol* **71**: 1739–1746.
- Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**: 1312–1316.
- Tibbetts, M.D., Zheng, L. and Lenardo, M.J. (2003) The death effector domain protein family: regulators of cellular homeostasis. *Nat Immunol* **4**: 404–409.
- Waldhuber, M., Emoto, K. and Petritsch, C. (2005) The *Drosophila* caspase DRONC is required for metamorphosis and cell death in response to irradiation and developmental signals. *Mech Dev* **122**: 914–927.
- Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., Agapite, J., *et al.* (2002) The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat Cell Biol* **4**: 445–450.
- Wu, Y., Parthasarathy, R., Bai, H. and Palli, S.R. (2006) Mechanisms of midgut remodeling: juvenile hormone analog methoprene blocks midgut metamorphosis by modulating ecdysone action. *Mech Dev* **123**: 530–547.
- Xu, D., Li, Y., Arcaro, M., Lackey, M. and Bergmann, A. (2005) The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in *Drosophila*. *Development* **132**: 2125–2134.
- Yan, N., Huh, J.R., Schirf, V., Demeler, B., Hay, B.A. and Shi, Y. (2006) Structure and activation mechanism of the *Drosophila* initiator caspase Dronc. *J Biol Chem* **281**: 8667–8674.
- Zieler, H. and Dvorak, J.A. (2000) 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* **97**: 11516–11521.