The Identification and Characterisation of Two

Novel Drosophila Caspases, DRONC and

DECAY.

by

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Abstract

Apoptosis is a process of active and programmed cellular death that serves an essential role during development and adult tissue homeostasis. Dysregulation of apoptosis may result in excessive cell death, which has been associated with various degenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease, and autoimmune disorders. In contrast, inefficient apoptosis has been linked to the development of many cancers. The identification of all the components involved in the control of apoptosis is therefore important for the understanding of aberrant apoptosis in disease and for subsequent therapeutic intervention.

Programmed cell death in metazoans is mediated by a family of cysteine proteases, termed caspases, which execute cell death through the cleavage of various cellular substrates after aspartate residues. A number of caspases have been described in both vertebrates and invertebrates. To date, fourteen mammalian caspases have been identified but only a few have been described in other organisms. The studies presented in this thesis make use of Drosophila as a simple model system for identification and characterisation of the molecules involved in apoptosis. At the commencement of this project, three caspases, DCP-1, DCP-2/DREDD and drICE were characterised in Drosophila melanogaster. Two other Drosophila caspases, DAMM and STRICA, have been recently cloned and characterised in our laboratory. The studies described in this thesis concentrate on the cloning and characterisation of the remaining two Drosophila caspases, DRONC and DECAY.

Caspases can be divided into two classes based on the presence of an amino-terminal prodomain that often contains protein-protein interaction motifs such as a caspase recruitment domain (CARD) or death effector domain (DED). The Class I prodomain containing caspases, are proposed to act as upstream activators of Class II caspases, which lack this domain. Activation of these latter caspases results in the cleavage of various cellular substrates and execution of the apoptotic pathway. There are several Class I caspases in mammals but only two, DCP-2/DREDD and STRICA, have been identified in Drosophila. In a search for CARD-containing Drosophila Class I caspases, a novel caspase, which we named DRONC, was identified. Initial characterisation of DRONC
demonstrated its ability to induce apoptosis when overexpressed in cultured cells, that could be prevented by the specific caspase inhibitors P35 and DIAP1. *dronc* is ubiquitously expressed in *Drosophila* embryos during early stages of development. In late third instar larvae, *dronc* mRNA is dramatically up-regulated in salivary gland and midgut, prior to histolysis of these tissues, by the steroid hormone ecdysone. These results suggest that DRONC is an effector of steroid-mediated apoptosis during insect metamorphosis.

The processing of DRONC during apoptosis was initially determined by *in vitro* incubation of DRONC with various active caspases and cell lysates. DRONC was found to be proteolytically cleaved from its 50 kDa pre-cursor form, into 38 kDa, 36 kDa and 18 kDa intermediates and a 14 kDa small subunit was detected *in vitro*. Further analysis of DRONC processing and activation *in vivo*, demonstrated processing of proDRONC early during apoptosis induced by various stimuli.

To determine the physiological function of DRONC, transgenic flies expressing *dronc* ectopically in the fly eye were generated. These flies displayed severe ablation of ommatidia and pigment cells in the eye. This phenotype was suppressed by expression of *p35, diap1* and *diap2* and enhanced by mutations in *diap1*. Halving the dosage of the *H99* gene complex (*reaper, hid* and *grim*) partially rescued the *dronc* eye phenotype, suggesting that these upstream activators are required for DRONC-mediated cell death. DRONC was shown to interact both genetically and biochemically with the CED-4/Apaf-1 fly homologue DARK. Furthermore, extracts made from *dark* homozygous mutant flies have reduced ability to process DRONC, suggesting that DARK is required to process DRONC. Finally, using RNA interference technique, it was shown that loss of *dronc* function in early *Drosophila* embryos results in a dramatic decrease in cell death, suggesting that DRONC is important for programmed cell death during embryogenesis. These results indicate that DRONC is a key caspase mediating programmed cell death in *Drosophila*.

In a database search to identify other *Drosophila* caspases, a novel Class II caspase, we named DECAY, was discovered, that shares a high degree of homology with mammalian Caspase-3. Ectopic expression of DECAY in cultured cells can induce
apoptosis. Low level of decay mRNA is ubiquitously expressed in Drosophila embryos during early stages of development and expression becomes somewhat spatially restricted in some tissues. During oogenesis, decay mRNA is detected in egg chambers of all stages, consistent with a role for DECAY in apoptosis of nurse cells. High levels of decay can also be detected in the salivary gland and midgut prior to pupal metamorphosis, implicating a role for DECAY in developmentally programmed cell death in Drosophila.

Biochemical analysis of recombinant DECAY demonstrated that this caspase has a substrate specificity similar to that of Caspase-3. Processing and activation of DECAY was detected during cycloheximide treatment of SL2 cells in vivo demonstrating that DECAY can be processed during apoptosis.

Most of the studies presented in this thesis have been published. The studies reported here and subsequent work from other laboratories have demonstrated that DRONC is a key initiator Class I caspase in Drosophila. The in vivo function of DECAY in apoptosis is not well understood, however the work presented here provides a framework for these studies in the future.
Statement

This thesis contains no material which has been accepted for any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by any other person, except where due reference has been made. I give consent for this thesis to be made available for loan and photocopying.

16/3/01

Dr. Leonie Quinn, from the laboratory of Dr. Helena Richardson (Peter MacCallum Institute, Melbourne) was responsible for the fly manipulations described in this thesis. Michelle Coombe, also part of Dr. Helena Richardson's group, assisted with dsRNA injections and Kathryn Mills assisted with fly crosses.
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To all, thankyou.
Publications


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>amc</td>
<td>7-amino-4-methylcoumaride</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor</td>
</tr>
<tr>
<td>bcl-2</td>
<td>B-cell lymphoma gene</td>
</tr>
<tr>
<td>BC-R</td>
<td>Broad complex receptor</td>
</tr>
<tr>
<td>BH (1-4)</td>
<td>Bcl-2 homology domains (1-4)</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus inhibitor repeats</td>
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<td>bp</td>
<td>base pair</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAD</td>
<td>Caspase activated DNase</td>
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<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
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<tr>
<td>Caspase</td>
<td>cysteinyl aspartate-specific protease</td>
</tr>
<tr>
<td>ced</td>
<td><em>C. elegans</em> cell death defective gene</td>
</tr>
<tr>
<td>ces</td>
<td><em>C. elegans</em> death specification gene</td>
</tr>
<tr>
<td>CrmA</td>
<td>Cytokine response modifier</td>
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<tr>
<td>DAMM</td>
<td>Death associated molecule related to Mch2</td>
</tr>
<tr>
<td>DAPAF-1</td>
<td><em>Drosophila</em> Apaf-1 homologue</td>
</tr>
<tr>
<td>DARK</td>
<td><em>Drosophila</em> Apaf-1-related killer</td>
</tr>
<tr>
<td>DBORG</td>
<td><em>Drosophila</em> Bcl-2 ortholog</td>
</tr>
<tr>
<td>DBOK</td>
<td><em>Drosophila</em> homologue of Bok</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>DEBCL</td>
<td>Death executioner Bcl-2 homologue</td>
</tr>
<tr>
<td>DECAY</td>
<td>Death executioner caspase related to Apopain/Yama</td>
</tr>
<tr>
<td>DED</td>
<td>Death effector domain</td>
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<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
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<tr>
<td>DIAP1/2</td>
<td><em>Drosophila</em> inhibitor of apoptosis 1 and 2</td>
</tr>
<tr>
<td>DISC</td>
<td>Death inducing signalling complex</td>
</tr>
<tr>
<td>DCP-1/2</td>
<td><em>Drosophila</em> caspase -1/2</td>
</tr>
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<td>DrICE</td>
<td><em>Drosophila</em> ICE</td>
</tr>
<tr>
<td>DREDD</td>
<td>Death related Ced-3/Nedd2-like protein</td>
</tr>
<tr>
<td>DROB</td>
<td><em>Drosophila</em> ortholog of Bcl-2</td>
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<td>DRONC</td>
<td><em>Drosophila</em> Nedd2-like caspase</td>
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<tr>
<td>DTT</td>
<td>1, 4-dithiothreitol</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EcR</td>
<td>Ecdysone receptor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>egl-l</td>
<td>egg-laying defective gene</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
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<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FLIP</td>
<td>FLICE inhibitory protein</td>
</tr>
<tr>
<td>fmk</td>
<td>fluoromethyl ketone</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GMR</td>
<td>glass minimal region</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
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<tr>
<td>HAC-1</td>
<td>Homologue of Apaf-1 / Ced-4</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine N’2-ethane sulphonic acid</td>
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<tr>
<td>hid</td>
<td>head involution defective gene</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>hsp</td>
<td>heat shock promoter</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis proteins</td>
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<tr>
<td>ICAD</td>
<td>Inhibitor of CAD</td>
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<tr>
<td>ICE</td>
<td>interleukin 1-β converting enzyme</td>
</tr>
<tr>
<td>IgG&lt;sub&gt;H/L&lt;/sub&gt;</td>
<td>immunoglobin G (heavy / light chain)</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>MOPS</td>
<td>3-[N-morpholino] propanesulfonic acid</td>
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<tr>
<td>MPD</td>
<td>minus pro-domain</td>
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<td>NAIP</td>
<td>neuronal apoptosis inhibitory protein</td>
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<td>nedd2</td>
<td>neural precursor cell expressed developmentally downregulated gene</td>
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<td>NP40</td>
<td>Nonidet P40</td>
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<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS(T)</td>
<td>Phosphate buffered saline (Tween 20)</td>
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<tr>
<td>PCD</td>
<td>programmed cell death</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RT-PCR</td>
<td>reverse-transcribed PCR</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>STRICA</td>
<td>serine / threonine rich caspase</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline (Tween 20)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGFR</td>
<td>Transforming growth factor receptor</td>
</tr>
<tr>
<td>TNF(R)</td>
<td>tumor necrosis factor (receptor)</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR associating death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR associating factor</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated nick end labelling</td>
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<tr>
<td>USP</td>
<td>ultraspiracle</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<td>WB</td>
<td>western blotting</td>
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Chapter 1

Introduction
1.1 Apoptosis

The physiological death of a cell is a sequentially orchestrated and programmed event that plays an essential part in embryonic development and tissue homeostasis within multicellular organisms. Kerr and colleagues (1972) defined the apoptosis concept as a process of cellular self-destruction that not only occurred in response to cytotoxic stimulation of cells but also during normal physiology and pathology of tissues. Apoptosis is characterised by a distinctive set of morphological changes, including cell shrinkage and condensation, DNA fragmentation and plasma membrane blebbing (Kerr et al., 1972; Wyllie et al., 1980; Earnshaw, 1995). Importantly, the plasma membrane retains its integrity and the fragmented dead cells are rapidly phagocytosed by neighbouring cells or macrophages, without leakage of cytoplasmic constituents which would otherwise induce inflammation (Kerr et al., 1972). This contrasts to cell death by necrosis, where organelle rupture results in leakage of cellular contents and induction of an inflammatory response (Kerr et al., 1972). It should be noted that necrosis occurs as a result of acute cellular injury and is not a programmed event essential for animal development. Apoptosis is also associated with various molecular changes such as internucleosomal DNA fragmentation (Wyllie et al., 1980), cleavage of cellular proteins crucial for structural maintenance (reviewed in Watters and Waterhouse, 1998) and, in some cases, mitochondrial swelling and cytochrome c release (reviewed in Kroemer and Reed, 2000). Apoptosis can be induced by a broad range of external stimuli, such as ionising radiation, cytotoxic drugs, serum deprivation or factor withdrawal, and can be observed in different cell types and tissues from various species. It can therefore be envisaged that the process of cell death is evolutionarily conserved, with various signalling molecules converging on a common death effector pathway.

Although the concept of apoptosis was described by Kerr and colleagues in 1972, the morphological features of cell death were first recognised back in the 19th century (reviewed in Majno and Joris 1995), but its importance had been neglected for many years. The importance of apoptosis has been well defined during normal development, and includes the sculpting of structures such as the removal of webbing between digits or neural
Programmed cell death is also vital for control in cell number, to provide a balance between the level of cell division and cell death. Furthermore, maintenance of tissue homeostasis relies on the elimination of abnormal, misplaced, non-functional or harmful cells by apoptosis. Examples of this include the removal of developmentally impaired cells such as self-reactive T and B-cells and the destruction of superfluous, damaged or aged cells (Ellis et al., 1991; Stellar, 1995). Apoptosis also plays a role in eliminating viral infected cells and may be triggered by the action of cytotoxic T-lymphocytes (CTLs), soluble factors such as tumour necrosis factor (TNF) and Fas ligand (Fas-L), or may result from a disturbance of the regulation of normal cell growth. Many viruses encode anti-apoptotic proteins, which function to prevent the death of virally infected cells, maximising replication and facilitating persistent infection (reviewed in Shen and Shenk, 1995).

It has become increasingly clear that the regulation of apoptosis is a fundamental process in normal physiology and inappropriate apoptosis has been linked to the aetiology of a number of human disorders (reviewed in Thompson, 1995; and Hetts, 1998). Diseases associated with excessive apoptosis include neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease as well as immunodeficiency and autoimmune disorders such as AIDS. In contrast many leukaemias and other cancers have been associated with insufficient levels of apoptosis (reviewed in Hetts, 1998; Nicholson, 2000). The ability to prevent excessive death by inhibiting activation of the pathway, or to target resistant cells for death through activation of the apoptotic machinery, is essential for the control of aberrant apoptosis. An understanding of the molecular regulation of apoptosis is therefore important for therapeutic intervention.

1.2 Molecular machinery of apoptosis

In the mid 1980's the nematode Caenorhabditis elegans made its debut as an important model system for apoptosis and has contributed significantly to knowledge of the regulation of cell death. Genetic studies in C. elegans led to the identification of 15 genes that are important for programmed cell death. Two of these genes, ces-1 and ces-2
(cell death specification) function in the initial steps of apoptosis by specifying the cells destined to die. A mutation in *ces-1* has been shown to prevent the death of specific neuronal cells, and the *ces-2* gene appears to prevent these deaths by negatively regulating *ces-1* (Ellis and Horvitz 1991).

The remaining cell death (*ced*) genes are involved in the execution of death and engulfment of dead cells (Hengartner and Horvitz, 1994b; reviewed in Liu and Hengartner 1999b) (Figure 1.1A). Several molecules important for the execution of cell death have been characterised and three of these, *egl-1* (egg laying defective), *ced-3* and *ced-4*, are required for the death of precisely 131 of the total 1090 cells produced during development (Sulston *et al*., 1983; Ellis and Horvitz 1986; Yuan and Horvitz 1990, 1992; Horvitz *et al*., 1994; Conradt and Horvitz 1998). In contrast, the cell death gene, *ced-9*, has been shown to antagonise the function of *ced-3* and *ced-4* and protects cells from dying (Hengartner and Horvitz 1992). In *ced-9* loss-of-function mutants, massive ectopic cell death leads to embryonic lethality (Hengartner *et al*., 1992). The cell death signalling pathway in *C. elegans* relies on the presence of EGL-1 protein which mediates cell killing by binding to and sequestering CED-9, such that CED-9 can no longer bind to and inhibit CED-4 (Shaham and Horvitz, 1996; Chinnaiyan *et al*., 1997; del Peso *et al*., 1998, 2000) (Figure 1.1B). Several studies have demonstrated the localisation of CED-4 as an important factor in its ability to induce CED-3 activation and cell death (James *et al*., 1997; Wu *et al*., 1997; Chen *et al*., 2000). Chen and colleagues (2000) demonstrated that CED-9 was responsible for the localisation of CED-4 to mitochondria, in all surviving cells. During cell death, EGL-1 expression induced translocation of CED-4 to perinuclear membranes (Chen *et al*., 2000). Furthermore, this membrane targeting allows CED-4 protein to interact with and activate CED-3, and consequently execute apoptosis (Chinnaiyan *et al*., 1997). The sequencing of the *C. elegans* genome has recently identified two additional CED-3-like molecules, *csp-1* and *csp-2*, but to date their functions in cell death have not been elucidated (Shaham 1998).

Once the death program has been executed, the final step is the engulfment of dying cells by neighbouring cells or phagocytes. Again, genetic studies in *C. elegans* have lead to the identification of 7 genes that are essential for rapid removal of these cells. Of
Figure 1.1 Programmed cell death in *Caenorhabditis elegans*.

A) Genetic studies in *C.elegans* led to the identification of 15 genes important for PCD. These genes are divided into four groups based on their order of activity. *egl-1, ced-9, ced-4, ced-3* and *ced-8* function in the execution of cell death and the remaining *ced* genes are involved in engulfment of dead cells (adapted from Liu and Hengartner, 1999b).

B) Apoptosis in *Caenorhabditis elegans* is mediated through activation of CED-3 by CED-4. This is initiated through binding and sequestering of CED-9 by EGL-1, thus preventing CED-9 from binding and inactivating CED-4 activity.
### A

<table>
<thead>
<tr>
<th>DEATH TO DIE</th>
<th>EXECUTION</th>
<th>ENGULFMENT</th>
<th>DEGRADATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ces-2</td>
<td>egl-1</td>
<td>ced-1</td>
<td>nuc-1</td>
</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td>ced-12</td>
<td></td>
</tr>
</tbody>
</table>

### B

- **EGL-1** interacts with **CED-9**
- **CED-9** and **CED-4** combine to form an **inactive form**
- The **active form** of **CED-4** is converted to an **active caspase**
- This caspase triggers **Cell death**
these genes, the cell death gene *ced-8* was later postulated to encode a protein which appears to function as a death effector, downstream of CED-3, as mutations in *ced-8* appear to slow the death process (Stanfield and Horvitz, 2000). The two genes, *ced-1* and *ced-2*, were the first to be isolated as engulfment mutants, characterised by the persistence of dead cells following embryonic cell deaths (Hedgecock et al., 1983). The remaining cell death genes, *ced-5*, -6, -7, -10 and -12 were similarly identified as effecting the engulfment phase of apoptosis (Ellis et al., 1991; Chung et al., 2000). Further mutational studies have demonstrated the presence of two independent pathways, one comprising *ced-1*, -6 and -7 and the other of *ced-2*, -5, -10 and -12 (Ellis et al., 1991; Chung et al., 2000). Several of these genes have recently been characterised and appear to belong to a signalling pathway that controls phagocytosis and cell migration. CED-2 is similar to the human adaptor CrkII, a tyrosine kinase signal transducer that interacts with CED-5 (human DOCK180 homologue), a protein involved in the process of extending the surface of an engulfing cell to envelop dead cells (Wu and Horvitz, 1998a; Reddien and Horvitz, 2000). CED-10 is similar to human GTPase Rac and is activated by CED-2/CEP-5 in a signalling pathway to promote cytoskeletal reorganisation and phagocytosis (Reddien and Horvitz, 2000). CED-6 was characterised as an adaptor protein that functions in a tyrosine kinase pathway within dying cells, and recent identification of human CED-6 protein has indicated the presence of a highly conserved signal transduction pathway across species (Liu and Hengartner, 1998, 1999a; Smits et al., 1999). *Ced-7* encodes an ABC transporter proposed to translocate adhesion molecules that mediate interaction between the dying and engulfing cell (Wu and Horvitz, 1998b). The precise mechanism of engulfment genes is still unclear, but mutational studies have outlined the importance of engulfment in the final stage of apoptosis (Hedgecock et al., 1983; Ellis et al., 1991). The death of a cell results in the expression of surface receptors, or phospholipids, that signal to cause cytoskeletal rearrangements and cell surface extension in the process of enveloping dead cells.

The first indication that the regulation of apoptosis in *C. elegans* and mammals is highly conserved arose from the observation that the human proto-oncogene, *bcl-2*, could functionally complement the *ced-9* mutation in *C. elegans* (Vaux et al., 1992b; Hengartner and Horvitz, 1994a). In fact the CED-9 protein and the recently characterised pro-death
molecule EGL-1, are both structural and functional homologues of the mammalian Bcl-2 family (Hengartner and Horvitz 1994a). In contrast to CED-9, EGL-1 shares homology with the Bcl-2 family of cell death activators such as BID, BIK, BAD and Harakiri (Conradt and Horvitz, 1998; section 1.5.2). The ced-4 gene encodes a 63kDa protein that is homologous to mammalian Apaf-1 protein, responsible for the cleavage and activation of a mammalian CED-3 homologue (Li et al., 1997; Zou et al., 1997). The mammalian CED-3 family of proteins, now termed caspases, has been shown to play a central role in the cell death program through their endopeptidase activity, leading to cell demise.

1.3 Caspases

The identification of mammalian ced-3 homologues confirmed the presence of an evolutionarily conserved apoptotic pathway. There is now a large family of mammalian proteases homologous to CED-3 (reviewed in Strasser et al., 2000), which have been termed 'caspases' and are numbered according to their order of identification (Alnemri et al., 1996, 1997). Caspases are defined as cysteiny1 aspartate-specific proteases, which mediate the biochemical and morphological changes associated with the effector phase of apoptosis. Currently 14 mammalian caspases have been identified (reviewed in Strasser et al., 2000).

Characterisation of the C. elegans cell death gene ced-3 lead to the identification of regions containing homology with the mammalian cysteine protease, interleukin-1β converting enzyme (ICE/ Caspase-1) (Yuan et al., 1993; Ceretti et al., 1992). The homology between CED-3 and Caspase-1 was the first implication that CED-3 may induce cell death in C. elegans by acting as a protease (Yuan et al., 1993). Overexpression of either ced-3 or Caspase-1 induces apoptosis in various cell lines that can be blocked by the anti-apoptotic proteins Bcl-2 and CrmA (Miura et al., 1993). Caspase-1 was initially isolated from monocyctic cells, its major function being the proteolytic cleavage and activation of the inflammatory cytokine interleukin-1β (IL-1β) (Thornberry et al., 1992). Loss-of function mutation of Caspase-1 does not exhibit any extensive apoptotic defects (Kuida et al., 1995; Li et al., 1995). The only noticeable deficits in caspase-1−/− mice are
the lack of production of IL-1β and a partial resistance of thymocytes to undergo Fas-mediated apoptosis (Kuida et al., 1995). Despite a suggested role for Caspase-1 in Fas-mediated apoptosis, caspase-1<sup>−/−</sup> mice do not display a lymphoproliferative (lpr) phenotype associated with a mutation in the Fas gene (reviewed in Nagata and Goldstein 1995). Caspase-1 and its closest relatives, Caspase-4, -5 and -11, have now been shown to be primarily involved in the processing of pro-inflammatory cytokines, which may promote recruitment of inflammatory cells to the site of cell suicide as an anti-viral defence (Vaux et al., 1994; Kang et al., 2000).

Shortly after the cloning and characterisation of ICE, a second CED-3 like protein was identified and termed Nedd2 / Caspase-2 (Kumar et al., 1994). Nedd2 was initially characterised as a neural precursor cell expressed developmentally downregulated gene within the murine brain (Kumar et al., 1992). Initial studies confirmed Caspase-2 as a death protease by the demonstration that ectopic expression induces apoptosis in most cell types and expression of anti-sense Caspase-2 in factor-dependent cells partially suppresses apoptosis induced by factor withdrawal (Kumar et al., 1994; Kumar 1995). The caspase-2 gene is also selectively transactivated following transient cerebral ischaemia in the adult murine brain, which may be responsible for the subsequent neuronal death (Kinoshita et al., 1997). On the other hand, caspase-2 is downregulated in ovarian follicular cells upon gonadotropin-dependent survival (Flaws et al., 1995). Caspase-2 knockout animals fail to show any overt phenotype which might be expected from inhibition of apoptosis (Bergeron et al., 1998), but further analysis of caspase-2 null mice has revealed an extra number of female germ cells indicating a role for caspase-2 during oocyte development. In addition, oocytes from these mice show resistance to doxorubicin induced apoptosis and caspase-2 null B-cells are resistant to granzyme B and perforin induced death. These data indicate a role for Caspase-2 in certain cell types and during cytotoxic lymphocyte mediated death (Bergeron et al., 1998).

Identification of the third caspase, Caspase-3 (CPP32/ Yama/ Apopain) revealed greater homology to CED-3 than either Caspase-1 or -2 (Femandes-Alnemri et al., 1994; Nicholson et al., 1995). Consistent with its function as a CED-3 homologue, Caspase-3 is able to induce apoptosis in cultured cells (Femandes-Alnemri et al., 1994). Caspase-3 and
its closest relatives, Caspase-6 and -7, appear to be responsible for later stages of cell death as they are able to induce cleavage of vital cellular proteins involved in cell maintenance, thereby contributing to the execution phase of apoptosis (reviewed in Nicholson, 1999). Although caspase-6 knockout mice do not display any overt phenotype, a loss-of-function in caspase-3 or -7 is embryonic lethal (reviewed in Shearwin-Whyatt and Kumar, 1999). In fact caspase-3 mutant mice display reduced embryo size and brain hyperplasia, implicating Caspase-3 as an important mediator of neuronal cell death in the developing brain (Kuida et al., 1996). Interestingly, the defect in neuronal apoptosis is similar to the presence of extra cells and phenotypic characteristics displayed by a ced-3 mutation in C. elegans (Ellis and Horvitz 1986) providing evidence for evolutionarily conserved biochemical cell death machinery.

1.3.1 Caspase structure

All caspases exist as inactive precursor molecules called zymogens, which require proteolytic processing for activation. Upon apoptotic stimulation, procaspases are processed to generate a large and small subunit which heterodimerize to form the active enzyme (Walker et al., 1994; Wilson et al., 1994; Rotonda et al., 1996). Mutations in the catalytic cysteine residue eliminates the catalytic activity and autoprocessing of most caspases and are therefore crucial in generating mature active caspases (Walker et al., 1994; Wilson et al., 1994). The prodomain of caspases appears to have a crucial role in assisting quaternary folding and subunit association for the formation of active enzyme. This has been shown for Caspase-1 and Caspase-2, which cannot undergo autocatalytic processing in the absence of the prodomain (Van Criekinge et al., 1996; Butt et al., 1998). In both cases the prodomain is absolutely essential for dimerization and subsequent autoactivation of these caspases.

Caspases can be divided into two classes based on the presence of this amino-terminal prodomain (Figure 1.2). Class I caspases contain long amino-terminal prodomains which are important for protease folding, activation and protein-protein interactions (Ahmad et al., 1997b; Duan and Dixit 1997; Muzio et al., 1996). Class I
Figure 1.2 The two classes of caspases.

Caspases are classed on the basis of the presence of an amino-terminal prodomain. Class I caspases contain long prodomains as represented on the left. Protein-protein interaction motifs within each prodomain are shaded; CARD domains are shown in blue, DED domains are represented as black hatched boxes. The large subunits are shaded green and smaller subunits are white. The prodomain of Csp-2a is shown truncated and is actually twice as long as the prodomain of Csp-1a. Proteins are not drawn to scale, although the catalytic large subunits do not vary extensively in size. Active site sequences are indicated, with Caspase-8, -9, -10, Csp-1 and Csp-2 varying from the conserved QACRG sequence.
<table>
<thead>
<tr>
<th>Class I</th>
<th>Class II</th>
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<tbody>
<tr>
<td><strong>C. elegans</strong></td>
<td><strong>CED-3</strong></td>
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<tr>
<td></td>
<td><strong>Csp-1A</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Csp-2A</strong></td>
</tr>
<tr>
<td><strong>Mammalian</strong></td>
<td><strong>Caspase-1</strong></td>
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<tr>
<td></td>
<td><strong>Caspase-2</strong></td>
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<td></td>
<td><strong>Caspase-13</strong></td>
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<td></td>
<td><strong>Csp-1B</strong></td>
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<td><strong>Csp-2B</strong></td>
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<td><strong>Caspase-3</strong></td>
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<td><strong>Caspase-7</strong></td>
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<td></td>
<td><strong>Caspase-14</strong></td>
</tr>
</tbody>
</table>

**Legend:**
- **DED**
- **CARD**
proteases appear to act upstream within the apoptotic pathway and act as signal transducers, being the first to be activated in response to various apoptotic stimuli (reviewed in Hengartner, 2000). The long prodomains of Class I caspases contain specific protein-protein interaction motifs, which are essential for binding to other molecules necessary for the induction of an apoptotic signal in the cell. The death effector domain (DED) of Caspase-8 and -10 mediates binding to other DED containing proteins (FADD/TRADD) and recruits these caspases to death receptor signalling complexes where they are activated (section 1.4.1). Similarly, the caspase-recruitment domain (CARD) present in the remaining Class I caspases mediates binding to other CARD containing adaptor molecules. It is unclear whether this domain mediates interaction to death receptors but several adaptor molecules have been identified that are able to bind to these caspases via CARD-CARD interaction. In the case of Caspase-1 and -2, the CARD domain is also required for homodimerization and subsequent processing and autoactivation of these caspases (Gu et al., 1995b; Van Criekinge et al., 1996; Butt et al., 1998). Caspase-1 is able to bind to the adaptor molecule CARDIAK/RIP2/RICK, a serine-threonine kinase, via CARD-CARD interaction, and this mediates activation of Caspase-1 upon binding (Thome et al., 1998; McCarthy et al., 1998b; Inohara et al., 1998). CARDIAK is able to associate with the TNFR associated factors, TRAF-1 and TRAF-2, leading to the activation of NFκB. The CARD of proCaspase-2 mediates binding to the CARD of the adaptor molecules RAIDD/CRADD (Ahmad et al., 1997b; Duan and Dixit 1997; Shearwin-Whyatt et al., 2000). The CARD domain of Caspase-9 mediates interaction with the CARD of Apaf-1 and this association is crucial for Caspase-9 activation (section 1.4.2).

Class II caspases contain only very short or absent prodomains and include caspases most closely related to CED-3/Caspase-3, and mammalian Caspases-6, -7 and -14 (Xue et al., 1996; Fernandes-Alnemri et al., 1994, 1995a, 1995b). These caspases are downstream effectors of the apoptotic pathway and become activated primarily through proteolytic cleavage by activated Class I caspases. Class II caspases target most of the cellular substrates, which undergo cleavage in apoptotic cells or induce DNA fragmentation (Casciola-Rosen et al., 1996; reviewed in Nicholson, 1999).
1.3.2 Substrate specificities of caspases

Evidence to suggest that caspases may function synchronously within death pathways comes from the findings that individual caspases are able to induce the processing and activation of themselves, or of other family members, either directly or indirectly (reviewed in Kumar and Lavin 1996; Kumar, 1999). A simplified cascade of caspase activation in mammalian cells can be envisaged, such that Caspases-2, -8, -9 and -10 act upstream within an apoptotic pathway and function to induce activation of downstream Caspases-3, -7 and -6, which appear to target several cellular proteins important for cell structure and viability (Nicholson, 1999). Many caspase members, in particular Caspase-1 and Caspase-2, are able to autoprocess and thus activate themselves when overexpressed in various cell lines (Butt et al., 1998; Colussi et al., 1998; Kumar and Colussi, 1999). In vitro, many caspases can also cleave each other in possibly the same pathway to cell death (Fernandes-Alnemri et al., 1996; Harvey et al., 1996, 1997b; Srinivasula et al., 1996; Xue et al., 1996).

Caspases have stringent specificity to cleave carboxyl-terminal to an aspartate residue in the P1 position of their substrates. The catalytic cysteine residue and residues making up the P1 aspartate binding pocket are highly conserved among all caspases, but residues lying near the P2-P4 sites are quite divergent suggesting a role for substrate specificity of each caspase member (Thomberry et al., 1997). Residues comprising the active site at the carboxyl-terminus of the large (~p20) subunit are also highly conserved and most caspases have the pentapeptide sequence QACRG, with only few caspases differing in one or more residue.

Based on the specificity of the optimal cleavage peptide sequence, members of the caspase family can be divided into three groups (Thornberry et al., 1997; Talanian et al., 1997). Group I caspases (-1, -4 and -5) prefer the sequence (W/L)EHD, which differs from the recognition motif of group II caspases (-2, -3 and -7) DE¿D, and that of group III caspases (-6, -8 and -9) (L/V)E¿D. The cleavage specificity of caspases has been demonstrated through use of specific synthetic peptide inhibitors of each caspase group. For example, Ac-DEVD-CHO effectively inhibits proteolytic cleavage activity of Caspase-3 and -6 (Ki=0.2nM), but not Caspase-1 activity (Ki=17nM). Similarly,
Ac-YVAD-CHO is a much less potent inhibitor of Caspase-3 activity (Ki=12mM compared to Ki of 0.76nM for Caspase-1) (Thornberry et al., 1992; Nicholson et al., 1995; Casciola-Rosen et al., 1996; Talanian et al., 1997).

Given that caspases within each group have almost identical recognition motifs, it is possible that they are either functionally redundant, have tissue specific or developmental specific expression, or may function in different pathways to cell death. The recognition sequence for Caspase-1 was initially identified as Tyr-Val-Ala-Asp (YVAD), which is present in the pro-form of IL-1β (Thornberry et al., 1992; Margolin et al., 1997). The finding that the preferred Caspase-1 tetrapeptide sequence is WEHD suggested the presence of alternate substrates other than IL-1β (Rano et al., 1997). To this end, interferon-γ (IFN-γ) inducing factor and proIL-8 have been identified as alternative substrates for Caspase-1 which implicates a role for Caspase-1 in the immune response (Akita et al., 1997; Gu et al., 1997).

Although the tetrapeptide recognition sequences of each caspase group differ in specificity, there is an absolute requirement for an aspartate residue in the P1 position for all caspase members. According to the crystal structures of Caspase-1 and -3, P2 appears to be solvent exposed, which explains the broad range of amino acids this position can accommodate (Walker et al., 1994; Mittl et al., 1997; Talanian et al., 1997). This excludes the preference of Caspase-9, which has a stringent requirement for a histidine residue in P2 (Thornberry et al., 1997). A second common feature of the tetrapeptide sequences is the requirement for a glutamate residue in P3. It therefore appears that the P4 residue is the major determinant of recognition specificity (Thornberry et al., 1997). Group I caspases prefer a large aromatic amino acid in P4, which is substituted for an aliphatic hydrophobic amino acid for Caspases-4 and -5, whereas an aspartate residue at this position is necessary for the activity of group II caspases (Talanian et al., 1997; Thornberry et al., 1997). Caspase-2 diverges from group II caspases in that it appears to require an extra hydrophobic P5 residue to recognise and efficiently cleave its substrate(s) (Talanian et al., 1997). In contrast, group III caspases can accommodate various amino acids with large aliphatic side chains.
The recognition motif for Group II and some Group III caspases resembles sites within many proteins important in cell maintenance or repair, such as poly (ADP-ribose) polymerase (PARP) (Kaufmann 1993; Lazebnik et al., 1994), DNA-dependent protein kinase (DNA-PK) (Casciola-Rosen et al., 1995; Song et al., 1996), sterol regulatory element binding proteins (SREBs) and U1-70kDa small ribonucleoprotein (Casciola-Rosen et al., 1994; Wang et al., 1995, 1996b). VEID, the recognition tetrapeptide for Caspase-6 is found in the nuclear protein lamin, which accounts for the collapse of nuclear structure that can be morphologically observed during apoptosis (Takahashi et al., 1996).

In fact, in the presence of the Caspase-3 peptide inhibitor Ac-DEVD-cmk, the activity of Group II caspases and cleavage of cellular substrates can be specifically inhibited (Mittl et al., 1997). In vitro studies have demonstrated the predominant presence of DEVD-afc (Caspase-3) and VEID-amc (Caspase -6) -like activity in apoptotic cells that accounts for the cleavage of PARP and nuclear lamins (Faleiro et al., 1997; Martins et al., 1997; Polverino and Patterson 1997). Other group II caspases cleave PARP with markedly different efficiencies suggesting they act indirectly on PARP by activating Caspase-3-like proteins (Gu et al., 1995a; Kumar and Harvey 1995; Margolin et al., 1997).

The cleavage of DNA at nucleosomal intervals is a hallmark of apoptosis. The mammalian instigator of apoptosis-induced DNA cleavage was isolated as DNA Fragmentation Factor 45 kDa subunit (DFF45) (Liu et al., 1997), or inhibitor of caspase activated DNase (ICAD) (Enari et al., 1998; Sakahira et al., 1998). The finding that Caspase-3 is essential for the activation of DFF45 to subsequently induce the fragmentation of DNA was the first evidence of a direct correlation between the activation of caspases to the final endonucleosomal cleavage of DNA. ICAD is a murine homologue of DFF45, which was shown to inhibit and sequester the caspase activated DNase, CAD in the cytoplasm (Enari et al., 1998). Upon apoptotic stimulation, Caspase-3 is able to process and activate ICAD, releasing CAD to enter the nucleus and fragment DNA (Enari et al., 1998; Sakahira et al., 1998). This finding has established a direct role for Caspase-3 in the execution of cell death.

Group III caspases appear to act as upstream effectors within the apoptotic pathway as they are able to recognise and cleave sequences present in other caspase members
(Caspase-3, -6 and -7) leading to their activation. Cytotoxic T-cell granule serine protease granzyme B shares the property of group III caspases and can activate several caspases which contain an IEPD motif (Caspase-2, -3, -6, -7, -8, -9 and -10) leading to apoptotic cell death during infection (Darmon et al., 1995; Harvey et al., 1996; Thornberry et al., 1997). Interestingly, some Group I and group II caspase recognition motifs are similar to their own activation sequence, indicative of their ability to self-cleave (Ramage et al., 1995; Kumar and Colussi, 1999). This supports the presence of an amplification cascade of proteolytic activation of caspases. It is therefore clear that individual caspase family members target specific cellular proteins and the identification of caspase substrates may elucidate their role in apoptosis in vivo. Table 1.1 outlines the preferred substrate recognition sequences of each caspase class member.

<table>
<thead>
<tr>
<th>Caspase group</th>
<th>Caspase members</th>
<th>Substrate specificity</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Caspase-1, -4, -5, -11, -12, -13</td>
<td>YVAD / WEHD, (W/L)EHD</td>
<td>pro-IL-1β / pro-IL-18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(W/L)EHD</td>
<td>Caspase-1</td>
</tr>
<tr>
<td>Group II</td>
<td>Caspase-2, -3, -7, -14</td>
<td>DEHD / VDVAD, DEVD</td>
<td>Golgin 160, PARP, DNA-PK, DFF, U1-70kDa, SREBP7 PARP</td>
</tr>
<tr>
<td>Group III</td>
<td>Caspase-6, -8, -9, -10</td>
<td>VEID, (L/V)ETD, LEHD</td>
<td>LaminA/C, Bid, Caspase-3, -4, -13, Caspase-3</td>
</tr>
</tbody>
</table>

Table 1.1 Caspase groups and substrate specificity.

Caspases are grouped on the basis of similarity in their optimal target recognition sequence (Talanian et al., 1997; Thornberry et al., 1997). Caspase-3 has many known substrates, only a few are listed here (reviewed in Nicholson, 1999).

1.4 Apoptosis signalling

The activation of caspases can be induced by a wide variety of apoptotic stimuli such that Class I caspases are processed and activate Class II caspases. It has become clear that apoptotic stimuli are able to induce death through different caspase-activating
cascades (reviewed in Hengartner, 2000). There are currently two known independent pathways that initiate activation of different Class I caspases and appear to converge to the activation of the same Class II caspases; a receptor-mediated death pathway and a pathway involving cytochrome c release from mitochondria (Figure 1.3).

1.4.1 Receptor-mediated pathway

In receptor-mediated apoptosis, the activation of caspases appears to be regulated by recruitment to various death receptors through adaptor molecules (FADD, CRADD). Death receptors belong to the tumour necrosis factor (TNF) receptor superfamily and include Fas, TNFR1, DR3, DR4 and DR5 (reviewed in Locksley et al., 2001). Apoptosis induction and caspase activation is best understood through the Fas and TNFR1 system (Figure 1.3). The Fas death receptor is highly expressed on the surface of mature lymphocytes and its over-expression has been associated with human immunodeficiency virus (HIV) or Epstein-Barr virus (EBV) infections (reviewed in Nagata and Goldstein, 1995). Binding of extracellular ligands (FasL and TNF respectively) to their receptors induces receptor trimerization. This in turn recruits an adaptor molecule FADD (Fas-associated death domain protein) / TRADD (TNFR-associated death domain protein) which associate with their respected receptors via death-domain (DD) interactions. The amino-terminal region of these adaptor molecules contains a DED that is able to recruit Class I Caspase-8 or -10 through DED interaction. Recruitment of Caspase-8 to this death inducing signalling complex (DISC) provides a localised concentration of Caspase-8 molecules, resulting in Caspase-8 oligomerization and proteolytic autoactivation (Chinnaiyan et al., 1995; Boldin et al., 1996; Fernandes-Alnemri et al., 1996; Muzio et al., 1996; Yang et al., 1998). Caspase-8 is activated first through cleavage of the prodomain, which releases Caspase-8 from the DISC and allows it to activate downstream caspases such as Caspase-3 -4 and -7 (Hirata et al, 1998). Active Caspase-8 is also able to cleave the Bcl-2 family pro-apoptotic member BID (Li et al., 1998b). Bid then acts by translocating to mitochondria and induces activation of Caspase-9 and -3, which may serve to amplify the death signal through a mitochondrial death pathway (section 1.4.2 below).
Figure 1.3 The mammalian apoptotic pathway.

Activation of caspases in mammals is mediated through either receptor signalling or mitochondrial signalling. Activation of death receptors, such as Fas and TNFR1, mediates binding of adaptor molecules, FADD and TRADD, via their death domains (DD). This leads to recruitment of Class I caspases (Caspase-8 and -10 in the case of Fas and TNFR1-induced death) to the death receptor via death effector domain (DED) interactions with the adaptor molecules. Once recruited, these caspases oligomerize and autoactivate, a process that is inhibited by cellular FLIP proteins. The resulting activated caspase can now process and activate Class II caspases, which cleave various cellular substrates in the execution phase of apoptosis. In response to other apoptotic stimuli such as factor deprivation, genotoxic damage and γ-irradiation, it appears that Class I caspases interact with adaptor molecules that are not necessarily attached to a receptor complex. A well described example is the activation of Caspase-9 by interaction with the CARD of Apaf-1. This process is dependent on the presence of cytochrome c and dATP, released from mitochondria, and can be inhibited by Bcl-2. Similarly a CARD-CARD interaction facilitates association of Caspase-2 with adaptor molecules such as RAIDD. Once activated, Class II caspases are activated to execute the apoptotic process. Diablo is a mitochondrial protein released upon apoptotic stimulation and induces cell death through sequestering IAP proteins, but it is unknown how it leads to activation of caspases. Inhibitory molecules are show in blue.
Death stimuli (factor deprivation, γ-irradiation, genotoxic damage)

Ligand (eg. FasL/TNF)

Death receptor (eg. Fas/TNFR)

Adaptor (eg. FADD, TRADD)

Class I pro-caspase

Class II pro-caspase

IAP

Caspase-9

Caspase-8

Active caspase

Substrate cleavage

Cell death
The importance of Caspase-8 in receptor-mediated apoptosis has come from loss of function studies. Caspase-8 null neuroblastoma cells confer resistance to Fas-mediated apoptosis (Varfolomeev et al., 1998). Caspase-8 inactivation has also been linked to neuroblastomas, suggesting it has a potential role as a tumour suppressor in the nervous system (Teitz et al., 2000). Caspase-8⁻/⁻ embryos die in utero and display impaired formation of cardiac muscles, abdominal haemorrhage and excessive hyperaemia in major blood vessels (Varfolomeev et al., 1998) emphasising a crucial role for Caspase-8 in PCD.

1.4.2 Mitochondrial pathway to cell death

The second pathway for the activation of caspases was only described in 1997 with the cloning of a putative mammalian CED-4 homologue, termed Apaf-1 (Apoptotic protease activating factor-1), which shares 22% identity and 48% similarity at the amino acid level with CED-4 (Zou et al., 1997). This novel 130 kDa monomeric protein contains a CARD at its amino terminus, and 12 carboxyl-terminal WD40 motifs. Apaf-1 appears to act as an adaptor molecule similar to FADD, primarily functioning to recruit Class I procaspase molecules, enabling them to self-process. Apaf-1 is able to bind to the CARD of Caspase-9 in the presence of dATP and cytochrome c, which is released from mitochondria, leading to the activation of Caspase-9 (Li et al., 1997). The WD40 motifs appear to act as a negative regulatory domain by preventing Apaf-1 oligomerization and interaction with Caspase-9. Deletion of the WD40 repeats makes Apaf-1 constitutively active and capable of processing proCaspase-9 in the absence of cytochrome c and dATP (Hu et al., 1998; Srinivasula et al., 1998).

Cytochrome c normally resides between the inner and outer mitochondrial membranes where it functions in the respiratory chain. The mechanism of cytochrome c release is still controversial and several alternative mechanisms have been proposed. Early stages of apoptosis have been shown to result in the collapse of the mitochondrial inner membrane potential (ΔΨm), resulting in the opening of permeability transition pores and release of cytochrome c or other apoptosis-inducing factors (AIF) into cytoplasm (reviewed in Kroemer and Reed, 2000). Cytochrome c re-localisation to cytosol interacts
with Apaf-1 WD40 repeats, which possibly induces a conformational change in Apaf-1, exposing its CARD domain and enabling interaction with the CARD of Caspase-9 (Hu et al., 1998; Kumar and Colussi 1999; Purring-Koch and McLendon 2000). Bcl-2 is able to prevent the release of cytochrome c from mitochondria, preventing caspase activation and apoptosis (Liu et al., 1996). The CED-4 like domain of Apaf-1 mediates oligomerization and binding of cytochrome c promotes multimerization of Apaf-1 which then recruits and activates Caspase-9 (Saleh et al., 1999; Zou et al., 1999; Qin et al., 1999). Once activated, Caspase-9 proteolytically activates Caspase-3. Mice deficient in cytochrome c die in utero, and cells lacking cytochrome c are defective in Caspase-3 activation and have increased sensitivity to apoptotic stimuli (Li et al., 2000a). Likewise, apaf-1 null mice are lacking in Caspase-9 and -3 activation (Hakem et al., 1998; Yoshida et al., 1998). Similar to the caspase-3 deficiency, mice homozygous for a deficiency in apaf-1 or caspase-9 display extensive abnormalities in the development of the nervous system, including enlargement of the brain, caused by superfluous cells within the germinal layer, and mice die before or soon after birth (Kuida et al., 1998; Hakem et al., 1998; Yoshida et al., 1998). The similarities in phenotypes of caspase-3 and caspase-9 deficient mice, and the lack of Caspase-3 activation in caspase-9−/− thymocytes, confirmed the notion that Caspase-9 activates the downstream Caspase-3 and this is important for neuronal cell death in the developing brain. Caspase-3−/− or caspase-9−/− mice however, do not display any substantial defects in apoptosis in other tissues. It therefore appears that Caspase-3 and -9 are essential for neuronal apoptosis, but not required, or functionally redundant in other tissues (Kuida et al., 1998; Hakem et al., 1998; Zheng et al., 1999, 2000).

It has been recently demonstrated that several heat shock proteins, HSP70, HSP27 and HSP90, prevent Apaf-1/Caspase-9 complex formation by binding to Apaf-1 or cytochrome c (Beere et al., 2000; Bruey et al., 2000; Pandey et al., 2000; Saleh et al., 2000). It is still unclear however, whether interaction with these chaperone proteins are directly associated with the apoptotic pathway. Another inhibitor of Apaf-1, named Aven, has been directly linked to inhibition of caspase activation by simultaneously binding BclxL and Apaf-1, thus interfering with Apaf-1 self-association and Caspase-9 activity (Nelson Chau et al., 2000). Apaf-1 is now known to belong to a family of mammalian
CED-4 like proteins, including Nod1/CARD4 (Inohara et al., 1999; Bertin et al., 1999) and DEPCAF1 (Hlaing et al., 2000) which are also able to interact with and activate Caspase-9. A fourth Apaf-1 family member, Nod2, appears to be primarily involved with NFκB activation pathway through interaction with the CARD containing protein RICK (Ogura et al., 2000). CARD4/Nod1 and DEFCAP1 are also able to induce NF-κB activation, but it is unclear whether any of these family members are essential for Caspase-9 activation.

The identification of multiple caspases indicates the need for stringent temporal and spatial regulation of cell death during development in higher organisms. Although the importance of many caspases in developmental PCD has been demonstrated through targeted mutagenesis, many loss-of-function mutations in caspases have not displayed any overt phenotypes or demonstrated ablation of all developmental cell deaths, suggesting a functional redundancy between some caspases or possible compensatory mechanisms. In fact, a deficiency in either caspase-3 or -9 can be compensated through activation of other caspases in a Bid-mediated mitochondrial pathway to death (Zheng et al., 2000). Bid normally translocates to mitochondria once cleaved by Caspase-8, and mediates cleavage and activation of Caspase-9 and -3 (Li et al., 1998b). In mice deficient for caspase-9 or -3, Fas-induced cleavage of Bid leads to the activation of other caspases (-2, -6 and -7) and apoptosis of hepatocytes (Zheng et al., 2000). Activation of these caspases is not normally seen in wild type mice, so some cells do appear to have compensatory mechanisms to induce apoptosis. Caspase-2 has been shown to translocate from mitochondria to cytosol during apoptosis (Susin et al., 1999). In addition, Apaf-1 can enhance activation of Caspase-2, which may explain why Caspase-2 can compensate for a deficiency in caspase-9, and induce activation of Caspase-6 and -7 (Yoshida et al., 1998). Given that caspase deficiencies can have severe consequences, the ability of other caspases to have compensatory functions ensures a 'fail-safe' mechanism in the prevention of pathologies associated with dysregulation of apoptosis.
1.5 Regulation of caspases

The ability of caspases to efficiently instigate the apoptotic program indicates the importance in tight regulation of their activity to prevent excessive death from occurring, which is evident in numerous neurodegenerative disorders. Regulation of the mammalian apoptotic pathway is becoming increasingly complex. Regulation of caspases is also achieved through differential expression of caspases in some tissues implicating possible tissue-specific or pathway specific functions. Alternatively spliced forms of caspases can also act as negative regulators of apoptosis through inactivation or inhibition of their functional caspase isoforms (Alnemri et al., 1995; Fernandes-Alnemri et al., 1994, 1995a,b; Boldin et al., 1996; Srinivasula et al., 1999; Wang et al., 1994; Kumar et al., 1997). Tissue specific regulation and expression levels of each isoform may also play a role in sensitivity or resistance to apoptosis induction. Some caspases also appear to be selectively activated by specific death-inducing stimuli indicating the presence of distinct regulatory elements for each caspase.

The most well defined and predominant mode of caspase regulation is through expression of various cellular proteins that are able to either prevent recruitment of caspases to death receptors, or death adaptor molecules, or are able to inhibit caspase catalytic activity. Each caspase regulator maintains a fine check on caspase activity and blocks their functions at different steps in the pathway to prevent aberrant cell death.

1.5.1 Viral inhibition of caspases

It is well documented that apoptosis is an important cellular defence against viral infection. This death is triggered through the action of cytotoxic T lymphocytes, tumour necrosis factor or Fas-signalling pathways (reviewed in Shen and Shenk 1995). Two viral proteins, cowpox virus cytokine response modifier (CrmA) and baculovirus P35, successfully inhibit apoptosis through direct irreversible inhibition of caspases (Gagliardini et al., 1994; Ray et al., 1992; Komiyama et al., 1994; Tewari et al., 1995b, c). CrmA was originally characterised for its ability to prevent the induction of an inflammatory response upon cowpox virus infection by inhibiting Caspase-1 (Palumbo et al., 1989; Ray et al.,
CrmA is cleaved by various caspases, which is essential for its inhibitory activity (Xue and Horvitz 1995). Caspase inhibition by CrmA also appears to be specific, for example, CrmA cannot protect against PCD when expressed in C. elegans because CED-3 is unable to cleave CrmA (Xue and Horvitz. 1995). Xue and Horvitz (1995) further demonstrated that if the CrmA, Caspase-1 cleavage site, is replaced with the CED-3 recognition sequence and expressed as a transgene in C. elegans, CrmA becomes a CED-3 substrate and protector against PCD, as determined by the extra cells present in the anterior pharynx of the nematode. Inhibitor kinetics has established that CrmA poorly inhibits various caspase members (eg. Caspase-3, -6, and -7) with the exception of Caspase-8, which it is able to inhibit with high affinity (Zhou et al., 1997b). This consequently supports the finding that CrmA can effectively inhibit Fas and TNF-mediated apoptosis (Tewari and Dixit 1995; Tewari et al., 1995a, Miura et al., 1995).

The role of a second viral gene, p35, in the inhibition of apoptosis, was originally defined through the characterisation of a baculovirus p35 mutant, which caused rapid premature death of Spodoptera frugiperda (SF-21) cells upon viral infection (Clem and Miller 1994). This p35 mutation, resulted in decreased viral infectivity in SF-21 cells (Clem et al., 1991) and indicated the importance of apoptosis as an anti-viral defence mechanism in invertebrates. To date, there is no known cellular homologue(s) of P35. An interesting feature of P35 is its ability to prevent apoptosis in phylogenetically diverse organisms and in response to a wide variety of apoptotic stimuli. Hay and colleagues (1994) demonstrated that transgenic expression of P35 is able to prevent developmental PCD in Drosophila and block X-irradiation induced death in the Drosophila eye. Expression of P35 in C. elegans prevents PCD in developing larvae and is able to rescue the lethal ced-9 mutant (Sugimoto et al., 1994). The mechanism of inhibition by P35 appears to lie in its ability to prevent the proteolytic activity of various caspases. Unlike CrmA, P35 seems to have a much broader specificity for caspases. Purified CED-3 and several mammalian caspases (-1, -2 and -3) are able to cleave P35 generating two subunits that are able to form a stable complex with caspases (Bump et al 1995). A mutation in the target aspartate in P35 eliminates this cleavage and its subsequent anti-apoptotic function (Bump et al., 1995; Xue and Horvitz 1995). The ability of P35 to inhibit caspases
elucidates its means of inhibiting apoptosis, mediated by various stimuli such as Fas, TNF, γ-irradiation, and serum/growth factor withdrawal (Rabizadeh et al., 1993; Beidler et al., 1995; Martinou et al., 1995; Dorstyn and Kumar 1997).

A second baculovirus protein, inhibitor of apoptosis OpIAP, was originally characterised as being able to functionally complement a mutation in p35 (Crook et al., 1993). The family of IAP proteins contain two conserved motifs, a carboxyl-terminal RING finger motif and a series of 1-3 novel repeats at the amino-terminus, termed baculoviral IAP repeats (BIR), which are important for their anti-apoptotic activity (Birnbaum et al., 1994). The importance of OpIAP in cell death has been demonstrated through its ability to inhibit apoptosis mediated by viral infection in cultured cells (Duckett et al. 1996). This inhibition of apoptosis is mediated through inhibition of caspase activity, further implicating caspases as important mediators of host cell apoptosis upon viral infection (Duckett et al., 1996; Dorstyn and Kumar 1997). OpIAP is able to block early processing of effector caspases by preventing the first cleavage between large and small subunits, whereas P35 appears to act later by blocking the further cleavage and maturation of the large subunit (LaCount et al., 2000).

A family of viral apoptosis-inhibitory proteins has been identified and designated v-FLIPs due to their ability to inhibit Caspase-8/FLICE, (Thome et al., 1997; reviewed in Wallach 1997). These viral FLIPs act by impeding recruitment of Caspase-8 to the Fas death receptor to prevent subsequent apoptotic signalling. The majority of γ-herpes viruses encode FLIPs, in order to successfully establish persistent infection through the inhibition of apoptosis. The ability of FLIPs to inhibit Caspase-8 is an essential requirement for the survival of these viruses, and confirms the importance of Caspase-8 in viral-mediated Fas-induced apoptosis.

1.5.2 Bcl-2 family of proteins.

The bcl-2 gene was first identified as being highly expressed in human B-cell lymphomas as a result of a t(14;18)(q32;q31) chromosomal translocation (Tsujimoto et al., 1984, 1985). This translocation brings the bcl-2 gene under the constitutive promoter for
Figure 1.4 The Bcl-2 family.

Structural comparison of members of the Bcl-2 family of proteins. Bcl-2 homology domains (BH1-BH4) are depicted. Anti-apoptotic and pro-apoptotic members have been divided, with all anti-apoptotic members containing a BH4 domain (shaded green). Transmembrane region (TM) is depicted at the carboxyl-terminus of protein. (adapted from Baliga and Kumar, 2001)
the immunoglobulin heavy chain gene and results in overexpression of Bcl-2 (Tsujimoto et al., 1984, 1986; Bakhshi et al., 1985). Upregulation of bcl-2 is a normal mechanism for positive selection of developing lymphocytes and is critical for the survival of mature peripheral B and T cells, but not essential for embryogenesis or lymphoid development (reviewed in Cory 1995; and Korsmeyer et al., 1992). The bcl-2 gene product was subsequently found to prevent PCD in cells induced by various stimuli, including withdrawal of growth factors, heat shock, UV-irradiation and cytotoxic drugs, but not from cytotoxic T cells and Fas-mediated death (Allsopp et al., 1993; Tsujimoto et al., 1989; Vaux et al., 1992a, 1992b). There are now over 20 Bcl-2 family members that have been characterised in mammals (Figure 1.4). All Bcl-2 family members contain 1-4 conserved motifs known as Bcl-2 homology domains (BH1-BH4). The anti-apoptotic proteins contain all four BH domains and include mammalian Bcl-2, Bcl-xL, and Bcl-w and C. elegans CED-9 (Boise et al., 1993; Gibson et al., 1996). In contrast, some pro-apoptotic members contain only three BH domains (BH1-BH3) including Bcl-xS, Bax, Bak and Bok (Chittenden et al., 1995; Farrow et al., 1995; Oltvai et al., 1993), or a BH3 domain alone, and are otherwise unrelated to any protein, including Bik, Blk, Bad, Bid, Bim and C. elegans EGL-1 (reviewed in Baliga and Kumar, 2001; Boyd et al., 1995; Wang et al., 1996a). Bcl-2 family members are able to heterodimerize via their BH3 domain, so the fate of a cell appears to be determined by the ratio of pro-survival to pro-apoptotic proteins, possibly via the same mechanism in which EGL-1 and CED-9 regulate each other (Oltvai et al., 1993, Yang et al., 1995, Minn et al., 1999).

Many Bcl-2 family members contain transmembrane anchor regions and reside on various membrane compartments such as the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope (Nguyen et al., 1994). Based on its NMR structure, Bcl-2 and Bcl-xL have been proposed to have pore forming ability on lipid membranes and regulate ion flow in and out of mitochondria (Kluck et al., 1997; Minn et al., 1997). The pro-apoptotic members, Bax, Bid, Bad and Bim translocate to mitochondrial membranes during apoptosis, and are predicted to induce cell death by inhibiting membrane bound Bcl-2 and Bcl-xL (reviewed in Gross et al., 1999; and Baliga et al., 2001). It has been proposed that Bcl-2 and Bcl-xL inhibit apoptosis by retaining mitochondrial potential,
preventing the opening of permeability transition pores and release of mitochondrial cytochrome c, which otherwise would induce caspase activation and apoptotic nuclear changes (Chinnaiyan et al., 1996; Shimizu et al., 1996b; Susin et al., 1996). Bax is able to induce release of cytochrome c from mitochondria both in vitro and in vivo, a process that is blocked by Bcl-xL (Kluck et al., 1999). Although in vitro evidence suggests that Bcl-2 family members form ion channels in the mitochondrial membrane to regulate release of mitochondrial proteins such as cytochrome c, there has been no in vivo demonstration of their pore forming abilities. The ability of Bcl-2 and Bcl-xL to localise to nuclear or endoplasmic reticulum membranes may also indicate that these proteins, function independent of cytochrome c. An alternative model for the biochemical regulation of Bcl-2 family proteins proposes that anti-apoptotic members bind and sequester cytosolic adaptor proteins to membranes (Strasser et al., 2000). Pro-apoptotic Bcl-2 members translocate to membranes during apoptosis where they induce a conformational change in Bcl-2 or Bcl-xL. Strasser and colleagues (2000) further propose that the converted anti-apoptotic family members can then interact with other anti-apoptotic members to induce conformational changes and mass release of adaptor proteins. These adaptor proteins can now bind and activate caspases in the cytosol. The conformational changes of anti-apoptotic Bcl-2 proteins may also influence mitochondrial membrane permeability and cytochrome c is likely to be released in this process.

The importance of Bcl-2 in apoptosis has been demonstrated in knockout studies. Although mice deficient for bcl-2 develop normally, they develop marked lymphoid apoptosis, excessive neuronal and melanocyte cell death and terminal kidney disease (Veis et al., 1994; Nakayama et al., 1994; Michaelidis et al., 1996). Mice deficient in bcl-xL die in utero due to excessive neuronal and erythroid cell death and their B cell development is impaired (Motoyama et al., 1995). Bcl-w−/− and bim−/− mice are essentially normally, but bcl-w−/− males are infertile and bim knockout suggests an important role for Bim in cytokine withdrawal-induced apoptosis as well as development and homeostasis of haemapoietic cells (reviewed in Baliga and Kumar, 2001). A loss in the pro-apoptotic gene bax results in accumulation of superfluous cells such as neurones, lymphocytes and germ cells (Knudsen et al., 1995; Brady et al., 1996; Deckwerth et al., 1996). A mutation
in \textit{bax} is also associated with human gastrointestinal cancers and some leukaemia’s (Rampino \textit{et al.}, 1997) and loss of \textit{bcl-2} is associated with the neurodegenerative disorder spinal muscular atrophy (SMA) (Iwahashi \textit{et al.}, 1997). \textit{Bax} is also upregulated in response to ischaemic injury which is concomitant with a down regulation in \textit{bcl-2} and \textit{bcl-xL} (Krajewski \textit{et al.}, 1995). \textit{Bak} knockout mice are essentially normal but when both \textit{bak} and \textit{bax} genes are simultaneously deleted, the effect is deleterious with only 10\% survival rate. \textit{Bak} \textsuperscript{-/-} / \textit{bax} \textsuperscript{-/-} mice that do survive develop multiple defects including interdigital webs, superfluous cells in the CNS and haemapoietic systems indicating these genes have overlapping roles in developmental apoptosis (Lindsten \textit{et al.}, 2000). Together these findings emphasise the importance of Bcl-2 family of proteins in apoptotic regulation.

\subsection*{1.5.3 \textit{Inhibitor of Apoptosis} proteins}

A mammalian IAP homologue, neuronal apoptosis inhibitory protein (NAIP), was identified through its association with spinal muscular atrophy (SMA), a disorder characterised by excessive motor neuron apoptosis during embryonic development. Individuals with this genetic disorder possess a non-functional NAIP protein that has an amino-terminal deletion of its first BIR domain, which may account for the excessive neuronal apoptosis (Roy \textit{et al.}, 1995). Overexpression of NAIP in cultured cells prevents apoptosis induced by numerous stimuli, which corresponds to NAIP acting as a negative regulator of motor-neuron apoptosis (Liston \textit{et al.}, 1996). A family of mammalian IAP proteins was subsequently identified based on their homology to baculovirus IAP and consists of \textit{xIAP} (MIHA/hILP), \textit{cIAP1} (MIHB), \textit{cIAP2} (MIHC) and Survivin (Duckett \textit{et al.}, 1996; Uren \textit{et al.}, 1996; Liston \textit{et al.}, 1996; Tamm \textit{et al.}, 1998), each containing a carboxyl-terminal RING finger domain and 1-3 BIR’s like their viral counterparts (Figure 1.5). Survivin is not directly involved in inhibiting apoptosis but seems to be primarily involved with chromosome segregation and cytokinesis (Li \textit{et al.}, 1998a, 1999). Expression of \textit{xIAP} and \textit{cIAP1} can block apoptosis induced by the overexpression of caspases and act through direct binding and inhibition of caspases (Uren \textit{et al.}, 1996; Deveraux \textit{et al.}, 1997; Dorstyn and Kumar 1997; Roy \textit{et al.}, 1997). It has been further
Figure 1.5 IAP family of proteins

Schematic representation of the structure of BIR-containing proteins showing a comparison between viral, *C. elegans* and mammalian IAPs. BIR (baculovirus IAP repeat), RING finger domains, CARD and UBC (ubiquitin conjugating) domains are depicted. (adapted from Hay, 2000).
demonstrated mammalian IAPs can directly bind to proCaspase-9 to prevent its processing and activation (Deveraux et al., 1998; Takahashi et al., 1998) and can also interact with active Caspase-9 in the apoptosome complex (Bratton et al., 2001; Srinivasula et al., 2001). In contrast IAPs appear to inhibit the activity of Caspase-3 and -7 by specifically interacting with their activated forms (Deveraux et al., 1997). Inhibition of Caspase-1, -6, -8 or -10 by IAPs has not been detected (Deveraux et al., 1998). IAPs can in turn be inhibited by and sequestered by the mammalian protein SMAC/DIABLO, a putative functional homologue of the Drosophila death proteins RPR, HID and GRIM (Du et al., 2000; Verhagen et al., 2000). SMAC/DIABLO is released from mitochondria during apoptosis and bind to IAP proteins, thereby destabilising their interaction with caspases, and leading to activation of the apoptotic pathway (Du et al., 2000; Verhagen et al., 2000).

Both cIAP1 and -2 contain CARD domains, which may facilitate interaction with CARD containing caspases and inhibit caspase activation by preventing their recruitment to adaptor molecules. These IAPs are also able to interact with TNFR2 associated factors (TRAF1 and TRAF2) to induce NFκB activation which in turn can activate transcription of several genes involved in cell proliferation and cell survival (Uren et al., 1996; Rothe et al., 1995). The balance between cell survival and cell death mediated by TNFR family members therefore appears to be dependent on the concentration of anti-apoptotic versus pro-apoptotic molecules within the cell.

### 1.5.4 Mammalian FLIP

Mammalian cellular FLIP acts as an apoptosis inhibitor like its viral counterparts (Irmler et al., 1997; reviewed in Wallach 1997). FLIP shares significant homology with the DED containing caspases (-8 and -10), but lacks the conserved regions required for protease activity. Several groups have isolated this protein, which has been variously named as FLIP (Irmler et al., 1997), Casper (Shu et al., 1997), CLARP (Inohara et al., 1997), Flame (Srinivasula et al., 1997), Cash (Goltsev et al., 1997), I-FLICE (Hu et al., 1997), MRIT (Han et al., 1997) and Ursurpin (Rasper et al., 1998). FLIP inhibits apoptosis induced by Fas or TNF, by the binding and inhibition of Caspase-8 and -10.
activity, thus preventing their recruitment to activated death receptors and subsequent activation of these caspases (Hu et al., 1997; Irmler et al., 1997; Srinivasula et al., 1997; Rasper et al., 1998). Several groups have also demonstrated the ability of FLIP to induce apoptosis through Caspase-8 activation (Han et al., 1997; Hu et al., 1997; Inohara et al., 1997; Shu et al., 1997) but its mechanism of action as a pro-apoptotic protein is not clear.

With the human genome sequence almost complete, it appears that the majority of important apoptotic regulatory proteins have been identified (Aravind et al., 2001). Although it is clear that some caspases function in the same pathway to apoptosis, their tissue or developmental specific functions have not been established. Analysis of the specific functions and regulation of caspases, is required for an understanding of the molecular basis of many disorders that arise from aberrant apoptotic events. Due to the complexity of in vivo experimentation in mammals, a need has arisen for a simpler model system in the analysis of the regulation of each caspase in specific pathways to death.

1.6 Drosophila melanogaster as a model to study programmed cell death

Drosophila melanogaster has only recently had an impact as a model system to study apoptosis. The fly is a powerful model to demonstrate the importance of apoptosis in defining positional information and embryonic patterning, a process that is poorly understood. As a genetic model, Drosophila can be easily maintained and manipulated. Mutant flies can be easily generated and detected by different eye colour, wing shape or bristle type. Furthermore, genetic manipulations of the fly are impossible to mimic in mammals and the regulation of complex pathways is more accessible to experimentation in the fly. Gene function is difficult to accurately define in mammals and knockout studies in mice have not always been able to define gene function. This is largely due to the fact that many proteins have redundant functions or result in embryonic lethality when deleted, making it impossible to analyse protein function in adults. Most importantly, the fly genome has been sequenced and essentially all the components that compose the fly cell
death machinery have recently been, or are being, identified (Rubin et al., 2000). The main components of the cell death machinery are highly conserved so findings can be recapitulated back into the mammalian system. The genetics of the fly is well developed and already making contributions to the understanding of crucial molecules involved in PCD.

The process of apoptosis can be closely followed during embryogenesis *Drosophila*, where cell death is used extensively to refine and sculpt all developing structures. Apoptosis is also essential in the fly to provide 'spatial precision' and organisation of cells across segmental boundaries, and for the development of nervous system and gut. The first signs of PCD within the fly can be seen during segmentation, where it is vital for cell fate specification within each of the 14 segments along the anterior/posterior axis (Klingsensmith et al., 1989). These individual segments are comprised of imaginal disc structures, which are ultimately responsible for giving rise to the principle structures and tissues in the adult organism (Sonnenfeld and Jacobs, 1995). PCD is also prominent during *Drosophila* oogenesis to generate mature fertile eggs, and the associated morphological changes can be observed under a microscope (reviewed in Buszczak and Cooley, 2000). Germline cell death is essential to eliminate nurse cells once they have nourished developing oocytes, and to eliminate mutant egg chambers that will not provide viable progeny, therefore preventing wastage of energy and dumping of nutrients into defective oocytes.

The most clear-cut example of cell death within the fly occurs during metamorphosis, where larval tissues undergo excessive reorganisation to establish adult structures. The morphogenetic changes that occur during pupation are mediated by the steroid hormone ecdysone (Jiang et al., 1997; reviewed in Baehrecke 2000). Pulses of ecdysone are associated with *Drosophila* developmental morphogenetic transitions and its major peak of activity occurs at the start of puparium formation, the start of metamorphosis (Jiang et al., 1997). Ecdysone acts through a receptor complex, comprising the nuclear hormone receptor family members EcR (ecdysone receptor) and Usp (ultraspiracle), which bind DNA and activate the transcription of several early puff genes (*BR-C, E74, E75, E93 and βFTZ-F1*) (Burtis et al., 1990; Segraves et al., 1990;
DiBello et al., 1991; Thomas et al., 1993; Hall and Thummel, 1998; Lee et al., 2000a). Each of these genes have been implicated in the regulation of PCD in specific larval tissues and act through regulating transcription of apoptosis regulatory genes in a tissue and stage-specific manner (reviewed in Thummel 1996; and Bachrecke, 2000). Mutations in BR-C prevent histolysis of larval salivary glands and imaginal disc elongation (Jiang et al., 1997). Ecdysone is crucial for controlling PCD of tissues such as larval salivary gland, midgut and for the degeneration of some muscle and neuronal cells and recent studies implicate ecdysone as a prime mediator of germline cell death (Truman, 1984; Thummel et al., 1996; Jiang et al., 1997; Buszczak and Cooley 2000). Ecdysone also regulates PCD of neural and muscle cells in the developed adult fly (Robinow et al., 1993).

In the adult fly, apoptosis is used extensively to further sculpt tissues, in particular the sculpting of Drosophila retina. The Drosophila eye is composed of tiny cells called ommatidia that are positioned to form a precisely ordered lattice network. The precision that gives rise to a highly ordered hexagonal lattice is mediated through spatially regulated PCD and is very susceptible to perturbation (Miller and Cagan, 1998). The Drosophila eye is a neat system to screen for genes involved in a number of cellular processes, in particular cell death, as the eye is not essential for fly viability. An increase in eye cell death can be directly visualised as the eye is markedly smaller and often results in an adult 'rough eye' phenotype, a distortion of the eye caused by disorganisation of ommatidia that is commonly associated with lack of pigment cells (Wolff and Ready, 1991; Miller and Cagan, 1998). Thus cell death in Drosophila is well defined and can be closely followed during development, making the fly a simple tool for the study of PCD regulation.

1.7 Drosophila death machinery

The evolutionary conservation of the cell death pathway is emphasised by the identification of conserved cell death genes in D. melanogaster required for apoptosis. Many early studies on PCD were carried out in Drosophila with the identification of three novel death genes absolutely essential for apoptosis.
1.7.1 Death genes

Studies of apoptosis in Drosophila embryos lead to the identification of a deficiency (H99) mapping to chromosome 3, 75C1-2, which is absolutely essential for programmed cell death during embryogenesis (White et al., 1994). Three genes were subsequently characterised in this region, termed reaper (rpr), hid (named after its mutant phenotype, head-involution defective) and grim, each an important mediator of developmental cell death (White et al., 1994; reviewed in Lee and Baehrecke 2000). Each of these genes are transcriptionally upregulated in response to various death-inducing stimuli and their gene products are able to induce apoptosis in cultured cells (White et al., 1994, 1996; Grether et al., 1995; Chen et al., 1996; Lee and Baehrecke 2000). Expression of rpr, hid and grim coincides with apoptosis events during development, particularly during early embryogenesis, and deletion of all three genes blocks apoptosis in Drosophila (White et al., 1994). During development of the embryo, rpr mRNA is upregulated in many dying cells, coinciding with all the morphogenetic changes in the head region (White et al., 1994; Nassif et al., 1998). Both rpr and hid are expressed during early embryo segmentation in dying cells of the midline and ectopic expression of both, together, is required for induction of midline cell death (Zhou et al., 1997a). In contrast, ectopic expression of grim on its own can efficiently induce cell death of the Drosophila CNS midline and grim can act synergistically with rpr and hid to enhance midline cell death (Wing et al., 1998). Expression of rpr and hid are also upregulated by ecdysone during metamorphosis in midgut and salivary glands (Jiang et al., 1997, 2000). Ecdysone regulation of neural death is concomitant with an upregulation of the ecdysone receptor isoform EcR-A and consequent accumulation of rpr and grim transcripts (Robinow et al., 1993, 1997). In response to DNA damage mediated by ionising radiation, rpr is transcriptionally upregulated (Nordstrom et al., 1996). This upsurge in rpr mRNA is mediated through the binding of p53 cell cycle check-point protein to its p53 radiation response element, providing the first direct link between induction of DNA damage to activation of apoptotic death molecules (Nordstrom et al., 1996; Brodsky et al., 2000).

The only structural similarity between RPR, HID and GRIM, resides in a 14-amino acid sequence at their amino terminus, referred to as the RHG motif, which does not
appear to be essential for their apoptosis-inducing activity (Wing et al., 1998). Rpr was the first gene identified in the H99 region and encodes a 65 amino acid cytoplasmic protein. RPR is able to induce apoptosis in a number of cultured cell lines via the activation of caspases (Pronk et al., 1996; Kondo et al., 1997). RPR-induced apoptosis can also promote release of cytochrome c from mitochondria (Evans et al., 1997). Recently, studies in Xenopus egg extracts demonstrated that RPR-induced apoptosis is mediated by interaction with Scythe, a 150 kDa Xenopus protein containing an amino-terminal ubiquitin-like domain (Thress et al., 1998). Furthermore, immunodepletion of Scythe from Xenopus egg extracts eliminates RPR-induced cytochrome c release, caspase activation and nuclear fragmentation (Thress et al., 1999). Scythe can also interact with GRIM and HID but it is unclear how Scythe mediates apoptosis via these proteins. A mammalian homologue of Scythe, termed Chap2, was recently identified as a ubiquitin-like protein that interacts with the ATPase domain of the Hsp70 protein Stch (Kaye et al., 2000). It is still unclear whether Chap2 functions in cell death, or in a ubiquitin mediated protein degradation pathway or most importantly whether Scythe mediates apoptosis via interaction with heat shock proteins in an Apaf-1 like pathway.

The hid gene product is a 410 amino acid protein rich in proline and serine residues (Grether et al., 1995). During development of the head region, hid is crucial for correct folding and formation of the frontal sac and hid mutant embryos contain extra cells in the head region due to lack of cell death, an involution defect (Abbott and Lengyel 1991; Grether et al., 1995). Expression of hid is not only confined to cells undergoing apoptosis, but is also detected in cells destined to survive. HID function is also linked to the Ras-MAPK (mitogen activated protein kinase) survival pathway, where MAPK is able to down-regulate hid transcript and is able to phosphorylate HID protein, therefore preventing HID-induced death (Kurada and White, 1998).

The grim gene was the last to be identified in the H99 region and is mapped between rpr and hid. The transcript for grim RNA is expressed at all stages of embryonic development, concomitant with the onset of cell death (Chen et al., 1996). GRIM encodes a 138 amino acid protein that can be processed by caspase-like activity. Similar to rpr and hid, ectopic expression of grim in the Drosophila eye results in a small eye phenotype due
to increased retinal cell death (Grether et al. 1995; Chen et al., 1996; White et al., 1996). Localisation of GRIM in cultured cells is mainly cytoplasmic, but upon apoptotic induction, GRIM, like HID, appears to localise to mitochondria (Claveria et al., 1998; Haining et al., 1999).

These proteins appear to act directly through inactivation of anti-apoptotic proteins (IAPs) and the activation of caspases, as their ability to induce apoptosis can be inhibited by DEVD tetrapeptide inhibitors or by P35 (Grether et al., 1995; Chen et al., 1996; White et al., 1996; Lee and Baehrecke 2000). In vivo, the induction of endogenous midline cell death by RPR and HID in Drosophila, is P35 inhibitable (Zhou et al., 1997a). Apoptosis by RPR, HID and GRIM can also be induced in mammalian cells, which is inhibited by Bcl-2/BclxL, indicating they all engage in a highly conserved pathway to death (Claveria et al., 1998; Evans et al., 1997; Haining et al., 1999). To date, there are no known mammalian orthologues of these proteins, although recently a putative mammalian functional homologue Smac/Diablo was identified, which promotes cytochrome c-dependent caspase activation through inhibition of IAPs (Chai et al., 2000; Du et al., 2000; Verhagen et al., 2000; Srinivasula et al., 2000). Due to the significant difference in the structure and sequence of Smac/DIABLO to RPR, HID or GRIM, it is still obscure as to whether it is a true counterpart of these death genes.

1.7.2 Drosophila caspases

The first insect caspase to be identified, S/Caspase-1, was isolated from Spodoptera frugiperda, as a target of the baculovirus anti-apoptotic protein P35 (Ahmad et al., 1997a). It was not until the late 1990s that Drosophila caspases were identified. At the commencement of this project very little was known about the cell death pathway in the fly and only three caspases, DCP-1, DCP-2/DREDD and drICE, had been identified in Drosophila. An important role for DCP-1 in developmental apoptosis has been defined through deletion studies and the functions of DREDD and drICE in cell death have only been established in the last two years. The recently completed genomic sequence of Drosophila demonstrated the presence of 4 other Drosophila caspases (reviewed in Kumar
and Doumanis, 2000), two of these, DAMM and STRICA are currently being characterised in our laboratory and the remaining two form the basis of the studies described in this thesis.

a) DCP-1

The first caspase dcp-1 (Drosophila Caspase-1), was isolated through polymerase chain reaction (PCR) using a Drosophila 4-8 hour embryo cDNA library (Song et al., 1997). DCP-1 encodes a 323 amino acid protein and has highest homology with CED-3, mammalian Caspase -3 and -6, and S. frugiperda Caspase-1. Based on the absence of an amino-terminal region, DCP-1 has been classified as a Class II effector caspase. Expression of dcp-1 transcript is high during embryogenesis, when extensive apoptosis is occurring but becomes more restricted in the embryo during later embryonic stages. The importance of DCP-1 in cell death has been demonstrated through a loss-of function mutation. A deletion of dcp-1 results in larval lethality and most homozygote flies die before the 3rd instar larval stage. Surviving larvae display normal central nervous system but have abnormalities including the presence of melanotic tumours and absence of imaginal discs and gonads (Song et al., 1997). This was the first indication that a caspase may have importance in preventing tumorigenesis. A loss of function mutation in dcp-1 leads to defective somatic and germ cell development and deficient transfer of nurse cell cytoplasmic nutrients to the developing oocyte, resulting in female sterility (Song et al., 1997; McCall and Stellar, 1998).

Song and colleagues (1997) further demonstrated that overexpression of DCP-1 is able to induce apoptosis and DNA fragmentation in cultured cells. Transgenic expression of truncated dcp-1, lacking the amino-terminal 28 amino acids, results in a small and rough eye phenotype which is further enhanced by expression of rpr and grim, suggesting these activators of cell death in the fly are able to activate DCP-1 in vivo (Song et al., 2000).
b) drICE

The second Class II Drosophila caspase to be identified, drICE (Drosophila ICE), was also isolated through PCR-based strategy. Like DCP-1, drICE has highest homology to CED-3, Caspase-3 and -6, and S. frugiperda Caspase-1 (Fraser and Evan 1997). The importance of drICE in apoptosis is ill defined due to the lack of loss-of-function mutations but drice transcript is expressed throughout development, with highest level detected during early embryogenesis when the majority of cell deaths are occurring (Fraser and Evan, 1997). Consistent with its role as a caspase, drICE overexpression sensitises Drosophila S2 cells to apoptosis and expression of an amino terminal truncated form induces rapid death of these cells (Fraser and Evan 1997). Apoptotic stimulation of Drosophila cells also induces processing of drICE (Fraser and Evan 1997; Fraser et al., 1997). Fraser and colleagues (1997) further demonstrated that immunodepletion of drICE from Drosophila SL2 lysates ablated all detectable DEVDase activity and that restoration of drICE could restore DEVD cleavage activity. This finding emphasised that drICE, functions similar to Caspase-3, as the primary DEVD-activator in these cells.

c) DCP-2/DREDD

A third Drosophila caspase DCP-2/DREDD, has highest degree of homology with Caspase-8 (Chen et al., 1998). DCP-2/DREDD contains two, weakly conserved amino-terminal DEDs in its prodomain region similar to Caspase-8 and -10 and a slightly divergent death inducing domain (DID) homologous to the DID in Drosophila FADD (dFADD). DREDD has been implicated as a Class I apical caspase based on its large amino-terminal prodomain. DREDD is processed and activated by the H99 gene products and can influence RPR and GRM-induced apoptosis (Chen et al., 1998). Cell death induced by ectopic expression of rpr, hid and grim can also be suppressed by halving the dosage of dredd, indicating that DREDD can modulate signalling by these death activators (Chen et al., 1998). Expression of dredd transcript is uniform in early embryos and accumulates in cells destined to die and overexpression of DREDD is also able to induce apoptosis in Drosophila SL2 (Chen et al., 1998).
DREDD is able to bind to a dFADD protein via DID-DID interaction. FADD is able to process DREDD and thereby enhances the cell death activity of DREDD (Hu and Yang 2000). Furthermore dFADD is highly expressed during early embryogenesis, at a time when dreed transcript is upregulated, so DREDD may mediate programmed cell death via a FADD-pathway in the fly, at least during embryogenesis. Sequencing of the genome has indicated the presence of a TNFR-like receptor in Drosophila so it is possible that DREDD acts through a TNF-like pathway in the fly (Aravind et al., 2001). To date it is not known whether dTNFR is involved in apoptosis and functions in a similar death pathway to mammalian TNFR, or whether dTNFR is involved in an NFκB survival response during infection.

A recent finding has demonstrated DREDD as an important mediator of the humoral immune response, particularly in resistance to bacterial infection (Leulier et al., 2000). The Drosophila immune system is primarily regulated by a family of NFκB/Rel transcription factors, which serve to induce the transcription of several antibacterial peptides in response to infection (Imler and Hoffmann 2000). There are two distinct pathways activated in response to infection that in turn lead to the activation of different Rel proteins. The immune response to fungal infection requires the Toll receptor-signalling pathway which, once activated, signals through two death domain containing proteins Tube and Pelle, resulting in release of the NFκB proteins (Dorsal or Dif) from their inhibitor Cactus (an IkB-like homologue). Dorsal and Dif are then translocated to the nucleus where they can activate transcription (Govind 1999; Imler and Hoffmann 2000). The second pathway is activated in response to bacterial infection and requires the Rel protein termed Relish, also a homologue of the mammalian NFκB precursor (Dushay et al., 1996; Hedengren et al., 1999). Similar to NFκB, Relish contains the conserved Rel homology domain (RHD) and a carboxyl-terminal IkB domain that inhibits it from translocating to the nucleus (Dushay et al., 1996). Relish is expressed highly during embryogenesis and is strongly upregulated in response to infection implicating an important role in both immunity and embryogenesis (Dushay et al., 1996).
Activation of an immune response leads to phosphorylation and proteolytic cleavage of Relish into two subunits; an amino-terminal RHD fragment translocates to the nucleus to induce transcription of several antimicrobial peptide genes, and a carboxyl-terminal IkB fragment that remains in the cytoplasm (Silverman et al., 2000; Stoven et al., 2000). Relish mutants are incapable of inducing transcription of antimicrobial peptides and, as a result, are increasingly susceptible to bacterial and fungal infection (Hedengren et al., 1999). In a genetic screen to identify immunocompromised mutants, the dredd gene was found to be a regulator of the Drosophila immune response (Elrod-Erickson et al., 2000). Mutations in the dredd gene resulted in impaired inducibility of antibacterial genes and inhibition of Relish processing, so that it could no longer translocate to nucleus and participate in transcription (Elrod-Erickson et al. 2000; Leulier et al., 2000; Stoven et al., 2000). It therefore appears that DREDD acts upstream of Relish and is important for its cleavage and activation during antibacterial immune response in the fly. The role of DREDD in the immune system may be to regulate apoptosis during inflammation, similar to mammalian Caspase-1 and the processing of IL-1β for synthesis of antimicrobial peptides. It is clear that DREDD plays an important role in the immune response and implicates a role for other caspases in physiological processes outside apoptosis.
d) DAMM

Recent identification of DAMM (Death Associated Molecule related to Mch2) in our laboratory, revealed sequence similarity with Class II Caspase-6 (Harvey et al., 2001). Expression of damm is low during early development and pupae stages whereas higher expression can be detected during adulthood. Higher expression of damm is also detected in 3rd instar larvae, particularly in larval salivary gland and midgut and in egg chambers of all stages, so DAMM may be involved in apoptosis of these various tissues. Preliminary biochemical studies have demonstrated that over-expression of DAMM in cultured cells can induce a low level of apoptosis that is inhibited by various viral and cellular apoptotic inhibitor proteins (Harvey et al., 2001). Harvey et al. (2001) also demonstrated that ectopic expression of damm in the Drosophila eye induces a rough eye phenotype that is further enhanced by \( \gamma \)-irradiation. It is still unclear as to where DAMM acts within the apoptotic pathway, but genetic analysis has demonstrated that a catalytic cysteine mutant of DAMM is able to suppress the eye ablation phenotype induced by hid, indicating a role for DAMM in HID-mediated apoptosis (Harvey et al., 2001).

e) STRICA

STRICA was identified through a database search and subsequently cloned from a Drosophila larval cDNA library (Doumanis et al., 2001). STRICA is a caspase comprising a serine/threonine rich prodomain and shares highest homology with the long prodomain caspases DREDD and Caspase-8. This novel caspase also contains an alternative QACKG active site sequence similar to DAMM. Doumanis et al., (2001) demonstrate that expression of strica is very low at all developmental stages with slightly higher expression detected in salivary gland and midgut, tissues that undergo histolysis during metamorphosis. Higher expression is also evident in nurse cells and developing oocytes indicative of a possible role in nurse cell death. Overexpression of STRICA induces death in Drosophila cells that can be inhibited by DIAP1 (Doumanis et al., 2001). The biochemical function of STRICA is still unclear but it has been shown to physically interact with both DIAP1 and DIAP2 (Doumanis et al., 2001).
The remaining two *Drosophila* caspases, DRONC and DECAY, constitute the studies in this thesis and are described in detail in the following chapters.

1.7.3 *Substrate specificities of Drosophila caspases*

Each *Drosophila* caspase comprises a catalytic active site that is slightly divergent from the consensus QACRG sequence. DCP-1 and drICE have a QACQG site, DCP-2 contains a QACQE catalytic active site, where as both DAMM and STRICA contain a QACKKG site. The alternative residues following the catalytic cysteine may simply be an indication of their substrate specificity. To date, the preferred substrate recognition site of these *Drosophila* caspases has not been defined. The Class II caspases DCP-1 and drICE have been categorised as group II caspases given their abilities to differentially process various cellular substrates at DEXD sites (Song et al., 2000) (Table 1.2).

Although the DEVD motif is optimal for group II caspases, DCP-1 also has preference for minor substitutions in the P2 residue (Song et al., 2000). In contrast drICE preferentially recognises a pentapeptide sequence DHTDA (DXXDA), indicating it may have alternative substrate specificity (Song et al., 2000). Furthermore, DCP-1 is able to process itself as well as drICE, which may act as an amplification point in the apoptotic pathway (Fraser et al., 1997). Both drICE and DAMM can be processed in cells undergoing apoptosis (Fraser et al., 1997; Harvey et al., 2001).

The cellular substrates for *Drosophila* caspases have not all been identified. DCP-1 and drICE can process P35, which accounts for P35 inhibition of these caspases (Song et al., 1997; 2000). Both DCP-1 and drICE are also able to process PARP and the nuclear cytoskeletal protein lamin Dm0, contributing to nuclear dismantling of the cell, and can be inhibited by DEVD-CHO and zVAD-fmk (Fraser and Evan 1997; Fraser et al., 1997; Song et al., 1997, 2000). The discovery of *Drosophila* caspase activated DNase (CAD) outlines the conservation and importance of DNA fragmentation in apoptosis. As in the mammalian system, CAD is able to bind to its inhibitor dICAD, which retains CAD in an inactive form. DrICE is able to process ICAD and release CAD from this complex, so that it can translocate to the nucleus to induce DNA fragmentation (Yokoyama et al., 2000).
### Table 1.2 Drosophila caspase classes and substrate specificity

<table>
<thead>
<tr>
<th>Caspase Class</th>
<th>Caspase members</th>
<th>Substrate specificity</th>
<th>Substrates</th>
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<tbody>
<tr>
<td>Class I</td>
<td>DCP-2</td>
<td></td>
<td>Relish</td>
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<tr>
<td></td>
<td>STRICA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td>DCP-1</td>
<td>DEXD, TETD</td>
<td>drICE, PARP, lamin Dm&lt;sub&gt;o&lt;/sub&gt;, P35</td>
</tr>
<tr>
<td></td>
<td>drICE</td>
<td>DEXD, DHTDA</td>
<td>dICAD, PARP, lamin Dm&lt;sub&gt;o&lt;/sub&gt;, P35</td>
</tr>
<tr>
<td></td>
<td>DAMM</td>
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Drosophila caspases are divided into two classes based on the presence of an amino-terminal prodomain. Optimal cleavage sequences and substrates for many caspases have not been determined.

### 1.8 Regulators of Drosophila caspases

The regulation of Drosophila caspases has only been established in the last year with the cloning of Apaf-1 and Bcl-2-like proteins in the fly. A proposed model for the regulation of caspases in the fly apoptotic pathway is displayed in Figure 1.6.

#### 1.8.1 Drosophila Apaf-1 protein

An investigation of the conserved function of CED-4 in different species found that ectopically expressed CED-4 in the Drosophila eye induces massive apoptosis of ommatidia, pigment and photoreceptor cells, that is associated with induction of Caspase-3 like activity, and can be partially rescued by P35 (Kanuka et al., 1999a). Overexpression of CED-4 in S2 cells induced extensive cell death that is accompanied by interaction with, and activation of drICE in a dATP-dependent manner (Kanuka et al., 1999a). These results demonstrated that CED-4 activity is conserved in Drosophila and that the fly contains a putative CED-4 like protein. A Drosophila CED-4 homologue or Apaf-1 related killer (DARK) was subsequently isolated and variously named DARK/DAPAF-1/HAC-1 (Kanuka et al., 1999b; Rodriguez et al., 1999; Zhou et al., 1999). DARK is a 170 kDa protein that contains all of the conserved regions seen in Apaf-1 and is able to interact with DREDD and induce apoptosis in Drosophila SL2 cells (reviewed in White, 1999). Additionally, an active site mutant of DREDD is able to abolish DARK-induced...
The current *Drosophila* model for caspase activation during apoptosis.

The pathway of apoptosis in *Drosophila melanogaster* is depicted. RPR, HID and GRIM act upstream in the pathway and are proposed to mediate activation of the Class I caspase DREDD either directly or through activation of DARK. DARK activates caspases in a cytochrome c dependent manner. Once activated, Class I caspases activate Class II caspases, which in turn process various cellular substrates in the execution phase of cell death. The inhibitor of apoptosis DIAP1, can block caspase activation and is itself sequestered and inactivated by RPR, HID and GRIM. The Bcl-2 family member Debcl is activated by RPR, HID and GRIM, and is able to activate the apoptotic pathway by acting upstream of DARK. It is not sure whether all apoptosis by RPR, HID or GRIM is mediated directly through Debcl.
Death stimuli

- RPR
- HID
- GRIM

Adaptor (dFADD)

**Class I pro-caspase** (DREDD)

**Class II pro-caspase** (DCP-1, drICE)

Active caspase

DIAP1

Active caspase

Substrate cleavage

Cell death

Debcl

Other functions (Relish cleavage)
cell killing (Rodriguez et al., 1999). The importance of the Drosophila CED-4 like protein has been demonstrated through a loss-of-function mutation, which ablates developmental PCD and leads to abnormal neuronal cell growth, development of melanotic tumours and extra cells in the eye (Kanuka et al., 1999b; Rodriguez et al., 1999; Zhou et al., 1999). Expression of dark is elevated during early embryogenesis, indicative of maternal deposition, and becomes restricted later in development to cells undergoing apoptosis (Zhou et al., 1999). Transcription of dark also appears to be upregulated during induction of apoptosis (Zhou et al., 1999).

The precise mechanism of action of DARK in the cell death pathway is still unclear but dark mutant flies have a reduced susceptibility to cell death mediated by ectopic expression of rpr, hid or grim (Rodriguez et al., 1999). Furthermore, halving the dosage of dark is able to suppress the eye ablation phenotype produced by ectopic dcp-1 expression (Rodriguez et al., 1999; Zhou et al., 1999). DARK has also been demonstrated to induce activation of DCP-1 and drICE in vitro (Kanuka et al., 1999b; Zhou et al. 1999).

Like Apaf-1, DARK appears to function in a cytochrome c / dATP-dependent manner. The addition of cytochrome c and dATP to Drosophila embryo extracts results in an increase in DEVD cleavage activity, which is significantly reduced in dark mutant extracts, indicative of lower DCP-1 and drICE activity in the absence of dark (Kanuka et al., 1999b). This, together with the finding that DARK can interact with cytochrome c, implicates cytochrome c as an essential mediator for the activation of DARK and downstream caspases (Kanuka et al., 1999b; Rodriguez et al., 1999). Kanuka and colleagues (1999b) characterised two isoforms of DARK, a long isoform as the major expressed form, and a shorter, active form that is able to bind and activate the caspases DCP-1 and drICE in a cytochrome c dependent manner. To this note, it is still controversial as to whether Drosophila cytochrome c is released from mitochondria like its mammalian counterpart (Liu et al., 1996; Kluck et al., 1997; Kanuka et al., 1999b). A recent hypothesis is that cytochrome c remains associated with mitochondria and simply changes conformation upon apoptotic stimulation to expose a 'hidden' epitope for binding various proteins and induction of caspase activity (Varkey et al., 1999). Further studies are required to associate the function of the fly cytochrome c to caspase activation.
1.8.2 Drosophila Bcl-2 family members

There are two Bcl-2-like proteins in Drosophila. One of these, Debcl/dBorg-1/dRob-1/dBok is a pro-apoptotic member (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Zhang et al., 2000). The function of the second Bcl-2 homologue, Buffy, is currently not understood. Both Debcl and Buffy contain three BH domains (BH1-BH3) and a carboxyl-terminal transmembrane domain that localises to mitochondrial membranes. Debcl induces cell death in Drosophila S2 cells, and ectopic expression of debcl in the Drosophila eye results in a severely ablated phenotype that can be partially repressed by P35 (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000). Genetic experiments demonstrated that Debcl can interact with DARK and IAP proteins but cannot interact with RPR, HID or GRIM suggesting that Debcl may mediate apoptosis through activation of DARK (Colussi et al., 2000).

1.8.3 Drosophila IAP proteins

The Drosophila homologues of IAP (DIAP1 and DIAP2) were among the first cellular IAPs to be isolated and were cloned on the basis of their homology to baculovirus IAP (Hay et al., 1995; reviewed in Hay, 2000). In a screen for dominant enhancers of GMR-rpr, hid and grim-dependent death, Hay and colleagues (1995) identified a lethal mutation in the thread (th) locus that acted as a strong enhancer, and cloning of thread identified it as an IAP-like protein (DIAP1). Overexpression of diapl and diap2 in the Drosophila eye is associated with inhibition of normally occurring cell death and results in the presence of extra pigment cells (Hay et al., 1995). Furthermore, overexpression of diapl or diap2 can suppress death due to over-expression of rpr, grim or hid. Zygotic loss-of-function diapl results in early morphogenetic arrest and embryonic lethality, indicating that DIAP1 is required for cell survival (Hay et al., 1995; Wang et al., 1999). The amino-terminal BIRs, conserved in all IAP proteins, were demonstrated to be sufficient, to prevent apoptosis in the Drosophila eye induced by X-ray irradiation (Hay et al., 1995). The carboxyl-terminal RING finger motif found in all IAPs has been implicated as a negative regulator of BIR death-inhibiting activity. A deletion of the
RING domain of DIAP1 results in greater protection from X-ray and hid-induced death in the fly eye (Hay et al., 1995; Vucic et al., 1998). DIAP1 and DIAP2 can also inhibit apoptosis induced by a variety of stimuli in cultured cells (Harvey et al., 1997a; Hawkins et al., 1998, 1999). The ability of DIAP1 to inhibit apoptosis appears to be through direct binding and inhibition of caspase activity, as DIAP1 mutant embryos have elevated levels of caspase activity (Hawkins et al., 1999; Wang et al., 1999). Both DCP-1 and drICE contain IAP target cleavage sites, and IAP cleavage by these caspases is necessary for its inhibitory activity (Kaiser et al., 1998; Huang et al., 2000).

DIAP1 and DIAP2 function is negatively regulated by RPR, HID and GRIM, which are able to promote apoptosis by binding and inhibiting DIAPs via their conserved amino-terminal 15 amino acids (Vucic et al., 1997, 1998; McCarthy and Dixit 1998; Goyal et al., 2000; Lisi et al., 2000). DIAP1 can inhibit DCP-1 activity in vitro, but in the presence of purified HID, caspase activity is elevated, supporting the notion that HID can induce apoptosis through inhibition and sequestration of DIAP1 (Wang et al., 1999). Decreasing the dosage of DIAP1 in the fly eye increases susceptibility to rpr- hid- and grim-induced death (Goyal et al., 2000). Furthermore, mutations of DIAP1 that cannot bind the H99 gene products, are more potent suppressors of rpr, grim and hid-dependent death in the fly eye (Goyal et al., 2000).

The function of DIAP2 in regulation of the death pathway is unclear, but DIAP2 has been shown to interact with STRICA and with the Drosophila Decapentaplegic (Dpp) type I receptor, Thick veins (Tkv), a TGFR family member (Oeda et al., 1998). This latter finding may indicate that DIAP2 inhibits cell death via Dpp pathway and downregulation of Jun N-terminal kinase (JNK) apoptosis signalling events.

The Drosophila homologue of Survivin named Deterin appears to be involved in apoptosis inhibition at least in cell culture (Jones et al., 2000). However, Deterin and another similar protein dBRUCE contain only BIR repeats and their function in cell death regulation is not clearly understood (Jones et al., 2000; Vernooy et al., 2000). Another possible mechanism of IAP inhibition of apoptosis may be through ubiquitination and degradation of pro-apoptotic molecules via the ubiquitin-like activity of their RING finger motifs (Yang et al., 2000).
1.9 Therapeutic aspects

The strong interest in the study of caspases and their regulation in apoptosis is their link with many disorders and potency as therapeutic agents against cancer. Preliminary work of inhibition or activation of caspases *in vivo* has had much positive outcome in the prevention of developmental apoptosis and several neurodegenerative disorders. Targeting of caspases themselves through specific antibodies has been potentially useful to induce self-activation and ultimately cell killing (Tse and Rabbitts 2000). Targeting regulators of apoptosis such as Bcl-2 through use of an antisense RNA fragment, has been shown to significantly reduce tumours in severe combined immunodeficient (SCID) mice. Similarly, use of antisense cFLIP has demonstrated success in treatment of carcinomas resistant to Fas-mediated killing, and antisense Survivin efficiently induces apoptosis in lung cancer cells, malignant melanomas and other cancers (reviewed in Nicholson, 2000). Use of recombinant TRAIL, which acts to induce apoptosis through ligation of the TNFR (DR4/DR5), has proven efficient in reduction of cancerous cells in various tissues (Nicholson, 2000). Clinical use of TRAIL has however been controversial due to its potential toxicity to hepatocytes (Nagata, 2000).

Caspase substrate inhibitors have also been useful as potential therapeutic molecules that can efficiently inhibit the exacerbation of infectious diseases such as meningitis and sepsis, and have improved the survival in several ischaemic models (liver, heart, intestine, renal and cerebral) (Cursio *et al.*, 1999; reviewed in Nicholson 2000). It was further demonstrated that VAD-fmk, can efficiently prevent fulminant liver destruction and death in mice mediated by administration of anti-Fas antibody (Ogasawari *et al.*, 1993; Rodriguez *et al.*, 1996). Hara *et al.* (1997) demonstrated the use of irreversible caspase inhibitors zVAD-fmk and zDEVD-fmk in protection from cerebral ischaemic injury and neural degeneration in mouse and rat. In addition, administration of zVAD-fmk can efficiently prevent the onset and mortality of the neurodegenerative disorder, amyotrophic lateral sclerosis (ALS) in mice (Li *et al.*, 2000b). Recent discovery of the chemical inhibitor, isatin sulfonamide, which specifically targets Caspase-3 and -7, has been demonstrated to inhibit chondrocyte death in osteoarthritic model systems and is a potential therapeutic drug for bone degenerative disorders (Lee *et al.*, 2000b).
Studies in *Drosophila* have already been invaluable in the understanding the molecular basis of pathogenic and degenerative disorders. The complex nervous system of *Drosophila* has become an attractive system for the study of neurodegenerative diseases, for example identification of *Drosophila* amyloid precursor protein (APPL) and presenilins in the fly has uncovered their possible function and effects in Alzheimer's disease (reviewed in Chan and Bonini 2000). Transgenic fly models for polyglutamine disease-induced neurodegeneration, expressing truncated forms of the human genes for Machado-Joseph Disease (MJD) and Huntington's disease, have demonstrated late-onset neurodegeneration and loss of photoreceptor neurons in the eye (Jackson *et al.*, 1998; Warrick *et al.*, 1998; reviewed in Chan and Bonini 2000). In addition, inhibition of caspases by ectopic expression of p35 in the eye of flies possessing a retinal degeneration disorder, inhibits apoptosis and subsequent blindness, thereby providing the first evidence that inhibiting caspase activation can prevent eye ablation *in vivo* (Davidson *et al.*, 1998). It is clear that the fly model has led to further understanding of several neurodegenerative mechanisms. These findings are significant steps forward to the treatment of such diseases and can potentially extend to the treatment of neurodegenerative disorders such as Alzheimer's, Parkinson's, SMA and even HIV-mediated AIDS.

The elaborate regulation of cell death during development is currently a topic of much research. A considerable amount of work is still required to understand the interactions between various death molecules in the execution of apoptosis both during embryogenesis and in adult tissue homeostasis. *Drosophila* contains many of the conserved molecules and pathways utilised by mammals in the regulation of cell death. Genetic strategies and manipulation of PCD in the fly is providing important information on the developmental and tissue specific action of caspases and how different death regulatory molecules can act in synchrony. Until the last few years, very little was known about the *Drosophila* death pathway, in particular the mechanism of caspase activation from an apoptotic stimulus and the activation of H99 proteins and to the organised dismantling of a cell. Research in the last two years, including the present study has contributed to our understanding of cell death in a simple model organism.
1.10 Aims

At the commencement of this study, an understanding of the pathway to apoptosis in *Drosophila* was only in its prime, and many of the apoptotic regulatory molecules had not been characterised. Extensive research in the last year lead to the identification of many important proteins involved in PCD in the fly, but the function of only three caspases had been described. An understanding of apoptosis regulation in a simple model organism such as the fly requires the identification of all molecules involved in the execution of cell death. Over the last three years, work in our laboratory has lead to the identification of four additional *Drosophila* caspases, two of these are described in the following chapters. This project specifically aimed to determine the following aspects:

1). To identify additional *Drosophila* caspases, particularly those that function as upstream or initiator molecules.

2). To biochemically characterise these caspases and to understand their function.

3). To establish the hierarchical position of these caspases in the genetic pathway by biochemical and genetic means.
Chapter 2

*Materials and Methods*
2.1 Manipulation of DNA

2.1.1 DNA quantification

DNA concentration was determined by measuring the absorption at 260nm on a spectrophotometer, assuming that an O.D.\textsubscript{260nm} of 1.0 represents 50\mu g/ml of DNA. Alternatively, DNA was separated by electrophoresis on agarose minigels and the intensities of the ethidium bromide stained bands were compared to known standard concentrations of DNA.

2.1.2 Electrophoresis of DNA

DNA was analysed by electrophoresis in agarose gels dissolved in Tris-acetate-EDTA buffer (TAE - 40 mM Tris-acetate, 1 mM EDTA) using 0.8-2% agarose depending on the size of DNA fragments to be retained. Prior to electrophoresis, 10x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to a final concentration of 1x and DNA loaded onto gels immersed in TAE buffer. Routine electrophoresis was performed at 80 -100 volts until the bromophenol blue dye front had run \( \frac{1}{4} \) down the gel. DNA was visualised by staining the gels in ethidium bromide solution (2\mu g/ml) for 10-30 mins and viewing under short-wavelength UV transilluminator (254nm) and photographed. Alternatively, DNA was visualised by scanning the ethidium bromide stained gel using FluorImager 595 (Molecular Dynamics) with a 610nm filter.

Standard molecular weight markers used were \textit{EcoRI} digested fragments of bacteriophage SPP1 and \textit{HindIII} digested bacteriophage lambda DNA (Geneworks).

2.1.3 Restriction endonuclease digestion

DNA was digested with the desired restriction endonucleases (Amersham Pharmacia Biotech or New England Biolabs) in a 10-50\mu l total volume comprising DNA, 1 unit restriction enzyme/\mu g DNA, 1x digestion buffer (specific for each restriction enzyme) with the addition of 0.1mg/ml bovine serum albumen (BSA) where required, in sterile H\textsubscript{2}O for 1-3 h at 37\degree C. Reactions were terminated by either heat inactivation at 65\degree C or 85\degree C (as per manufacturer protocol) or by addition of DNA loading dye.
2.1.4 Purification of DNA fragments

a) BRESA-CLEAN™

DNA was subject to electrophoresis as above, and the appropriately sized bands were excised from the ethidium bromide stained agarose gel under low energy ultra-violet irradiation. The weight of the excised band was determined and 3 volumes of BRESA-SALT™ solution added. The agarose was dissolved at 55°C for 5 min and mixed into solution. DNA already in solution was mixed directly with 3 volumes of BRESA-SALT™. To this DNA/salt mix, BRESA-BIND™ solution (5μl plus 1μl/μg DNA) was added and incubated at room temperature for 5 min with frequent gentle inversion to maintain BRESA-BIND™ in suspension. The BRESA-BIND™/DNA complex was pelleted by centrifugation at 9000g for 20 sec and the pellet washed twice with BRESA-WASH™ solution in a volume equivalent to that of the BRESA-SALT™ used in step 1. After the final wash, supernatant was aspirated and all traces of BRESA-WASH™ removed by drying at 55°C. DNA was recovered by resuspending the BRESA-BIND™/DNA complex in sterile H₂O equivalent to twice the volume of the BRESA-BIND™ matrix used and incubated at 55°C for 5 min. BRESA-BIND™ was pelleted by centrifugation for 1 min and the supernatant containing DNA was immediately transferred to a clean microfuge tube.

b) Phenol/chloroform extraction

DNA solution was made up to at least 200μl and an equal volume of phenol/chloroform (1:1) was added and shaken vigorously for 30 seconds. The aqueous and solvent layers were separated by centrifugation at 9000g for 1 minute. The upper, aqueous layer was removed and again extracted with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 volume 3M sodium acetate (NaAc) pH 4.6 and 2 volumes of 100% ethanol and incubation on ice for 1 h. DNA was pelleted by centrifugation at 13000rpm 15 min, 4°C, washed with 70% ethanol and resuspended in 10μl - 30μl sterile H₂O. In the case of very low concentration of plasmid DNA or PCR
product, 1μl of glycogen (Roche) was added prior to ethanol precipitation to minimise DNA loss.

2.1.5 DNA fragment end-filling

Restriction digested DNA with 3’ overhangs was end-filled by treatment with 1 unit Klenow (Amersham Pharmacia Biotech) and 0.2mM dNTP mix in 1x One-Phor-All Buffer Plus (Amersham Pharmacia Biotech) and sterile H2O for 30 min at 37°C. DNA was purified by phenol/chloroform extraction or BRESA-CLEAN™.

2.1.6 Kinase treatment of DNA

DNA fragments were phosphorylated by treatment with 1 unit of T4 DNA polynucleotide kinase (T4 PNK) (Amersham Pharmacia Biotech) and 0.5 mM ATP for 30 min at 37°C. Following kinase treatment, DNA was purified as above.

2.1.7 DNA dephosphorylation

To remove 5’ phosphate groups from restriction endonuclease digested DNA, DNA was treated with 1 unit of calf intestinal phosphatase (CIP) (New England Biolabs) in Buffer 3 (New England Biolabs) at 37°C for 30 min and purified as above.

2.1.8 DNA ligation

Ligation reactions were carried out in a 10 μl volume containing 1 Weiss unit T4 DNA ligase (Amersham Pharmacia Biotech), 1x ligation buffer (50mM Tris-Cl pH 7.6, 10mM MgCl2, 10mM DTT, 50μg/ml BSA, 1mM ATP), and DNA vector molecule ratio of 5:1. Reactions were allowed to proceed at 4°C overnight.

2.2 Transformation of Chemical Competent Cells

2.2.1 Preparation of E. coli competent cells

Five ml ψ broth (ψb - 20g/L Bacto-trypotpe [Difco], 5g/L Bacto-yeast extract [Difco], 5g/L MgSO4, and pH7.6 with KOH) was inoculated with a single bacterial colony and then grown overnight at 37°C with shaking. This culture was subcultured
1/20 into 100ml of pre-warmed yb and grown at 37°C with vigorous shaking until O.D.600nm reached 0.5-0.6. Cells were chilled on ice for 15 min and pelleted at 6000 rpm, 5 mins, 4°C. Medium was completely aspirated and cells resuspended in 40ml cold Tfb I (30mM KOAc, 100mM KCl, 10mM CaCl2·2H2O, 50mM MnCl2·4H2O, 15% glycerol adjusted to pH 5.8 with 0.2M acetic acid and filter sterilised). Cells were incubated on ice for 5 min. Cells were pelleted by centrifugation as above and resuspended in 4ml Tfb II (10mM MOPS, 75mM CaCl2·2H2O, 10mM KCl, 15% Glycerol adjusted to pH 6.5 with 0.5M KOH and filter sterilised). Incubated cells on ice for 5 min and transferred 50μl aliquots into pre-chilled 1.5ml microfuge tubes on dry ice. Stored all aliquots at -70°C.

2.2.2 Transformation of cells

Chemically competent E. coli bacteria were thawed on ice for 10 min. 4μl of ligation reaction was added to cells and incubated on ice for 20 min. Cells were heat shocked at 42°C for 90 seconds and cooled on ice for 1 minute. 1ml SOC medium (2% (w/v) bacto-tryptone [Difco], 0.5% (w/v) bacto-yeast extract [Difco], 0.05% (w/v) NaCl 0.25 mM KCl pH7, 10mM MgCl2, 200mM glucose, pH 7.0) was added and the culture incubated at 37°C for 1 h. Cells were pelleted by centrifugation, resuspended in 100μl SOC medium and plated onto agar plates (Luria broth + 15g/L Bacto-agar [Difco]) containing the appropriate antibiotics for vector encoded resistance (ampicillin 100μg/ml, kanamycin 25μg/ml or chloramphenicol 34μg/ml). In the case of vectors encoding the genes required for β-complementation, 4μl of 1M isopropylthio-β-D-galactoside (IPTG) (Progen) and 40μl of 20mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Progen), were spread onto plates prior to plating of the transformants. Plates were incubated at 37°C overnight.

2.2.3 Screening transformed cells by colony cracking

In the case of screening for vectors that did not allow for blue/white colour selection, colony cracking was employed to screen large numbers of transformants for plasmids containing the desired DNA inserts. Single transformed colonies were picked, spotted onto a grided agar plate and then transferred into a microfuge tube containing 15μl
cracking solution (50μM NaOH, 0.5% SDS, 5μM EDTA, 0.1% bromophenol blue). Bacterial colonies were lysed, by heating to 65°C for 15 min. Samples were loaded onto 1% agarose gels and electrophoresed at 40 Volts with the level of TAE just below the surface of the gel until DNA had migrated into the gel, after which TAE was added to cover the gel and electrophoresis continued at 100 Volts. Colonies containing positive clones were then further analysed by comparing plasmid mobility to that of empty vector.

2.3 Purification of Plasmid DNA from Bacterial Cultures

2.3.1 Small scale plasmid purification

Single transformed bacterial colonies were inoculated into 2ml LB containing the appropriate antibiotic (see section 2.2.2) and grown overnight at 37°C with shaking. 1.5ml of overnight bacterial cultures was transferred to a microcentrifuge tube and cooled on ice for 15min. The culture was pelleted and well in 100μl ice cold buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100μg/ml RNase A). Cells were lysed by the addition of 150μl buffer P2 (200mM NaOH, 1% SDS). Chromosomal DNA and protein was precipitated by mixing lysate with 100μl buffer P3 (3M KAc pH 5.5). Lysates were cleared by centrifugation at 9000g for 5 min and clear supernatants removed to clean microfuge tubes. DNA was precipitated by the addition of 2 volumes (600μl) 100% ethanol, 0.1 volume (30μl) sodium acetate pH 4.6 and incubation on ice for 20 min. DNA was then pelleted at 9000g for 15 min, 4°C and resuspended in 100μl sterile H2O.

2.3.2 Large scale plasmid purification

Larger scale plasmid DNA extraction and purification was carried out using BRESApure plasmid Midi Kit (Geneworks). Overnight bacterial cultures (50-100ml) were pelleted at 5000g and pellets resuspended in 4ml ice cold buffer BPR containing 100μg/ml Rnase A. Bacterial cells were lysed by addition of 4ml buffer BPL and incubated at room temperature for 5 min. Chromosomal DNA and protein was precipitated by addition of 4ml buffer BPN and mixed by gentle inversion. Lysates were centrifuged at 27000g for 30 min and supernatants transferred to BRESApure column,
which had been pre-equilibrated with 4ml buffer BPQ, and allowed to flow through. The columns were washed with 2x 10ml buffer BPW and DNA was eluted with 5ml buffer BPE. DNA was precipitated by addition of 0.7 volumes of isopropanol and pelleted by centrifugation at 15000g for 30 min at 4°C. The supernatant was carefully removed and DNA pellets air-dried and resuspended in 400μl sterile H2O. DNA solution was transferred to a 1.5ml microfuge tube and further precipitated by the addition of 0.1 volume 3M NaAc, pH 4.6 and 2.5 volumes of 100% ethanol and incubated on ice for 60 min. DNA was pelleted by centrifugation at 9000g, 15 min, 4°C, washed with 70% ethanol and resuspended in 100μl sterile H2O. The concentration of plasmid DNA in solution was measured as described in section 2.1.1.

2.4 Isolation and Purification of Genomic DNA

2.4.1 Mammalian DNA isolation

1x10⁷ cells were washed twice in PBS and pelleted at 15000g, 5 min, 4°C. Cells were resuspended in 500μl lysis buffer (100mM Tris-Cl pH 8.5, 5mM EDTA pH 8.0, 200mM NaCl, 0.2% SDS, 100μg Proteinase K/ml) and incubated at 37°C with shaking for 3-4h or until solution has cleared. DNA was precipitated by the addition of 1 volume of isopropanol and the sample mixed gently. The white aggregate precipitate (DNA) was lifted using a disposable tip and transferred to a clean microfuge tube. DNA was resuspended in 100-200μl TE (10mM Tris-HCl, 0.1mM EDTA pH7.5) and dissolved by incubation at 55°C for 1 h with agitation, or overnight at 37°C. 10μg of DNA was used for Southern Blotting procedures.

2.4.2 Drosophila genomic DNA isolation

Approximately 20 flies were homogenized in 100μl ice cold 0.1M Tris-HCl pH9/0.1M EDTA. To this, 100μl 2% SDS (pre-warmed to 65°C) was added and lysates incubated at 65°C for 1 h. After cooling samples to room temperature, 42μl 5M Kac pH5.5 was added and incubated on ice for 30 min to precipitate proteins. Pelleted precipitate by centrifugation at 9000g for 10 min and transferred supernatant to a clean
microfuge tube. Lysate was incubated on ice for 10 min and cleared again at 9000g, 10min. Supernatant was transferred to a clean tube and volume made up to 500μl with sterile H₂O. Phenol chloroform extracted samples twice and digested RNA by addition of 20μg/ml RNase A at 37°C for 10 min. Following a final phenol chloroform extraction, DNA was precipitated with an equal volume of isopropanol and incubated at RT for 10 min. DNA was pelleted at 9000g, 20 min, washed with 70% EtOH and resuspended in 20μl sterile H₂O, overnight at 4°C. 10μg Drosophila genomic DNA was digested for Southern Blotting.

2.5 Southern Blotting

2.5.1 Genomic DNA digestion and electrophoresis

A total of 10μg genomic DNA was digested in a volume of 40-50μl at 37°C for 3 h as described in section 2.2.3. An additional 1unit of enzyme and 3μl appropriate NEB buffer was added and the reaction incubated for a further 4 h at 37°C. DNA loading dye was added to each reaction and samples electrophoresed through a 0.8% agarose gel at 15-20V overnight in TAE buffer. After the gel was photographed under UV light, DNA was denatured in 0.5M NaOH, 1.5M NaCl for 1h with gentle shaking. The agarose gel was rinsed in distilled water and then neutralised in 1M Tris-HCl pH7, 1.5M NaCl for 30 min with gentle agitation. Neutralisation solution was changed and the gel washed for an additional 30 min. The DNA was then transferred to nylon membrane.

2.5.2 Southern transfer

Genomic DNA from gels was transferred onto Biodyne nylon transfer membrane (Pall) by capillary action overnight (as described in Maniatis et al., 1989). The gel was placed well-side down onto 3mm Whatman paper soaked in 20x SSC (175.3g/L NaCl, 88.2g/L sodium citrate, pH7). Biodyne membrane was cut to the size of the gel and carefully placed on top of the gel, followed by 3 sheets of 3mm Whatman filter paper and a large stack of absorbent paper towel. The stack was weighed down and left to transfer
overnight. The next day, membranes were cross-linked at 254nm shortwave-length UV using an Ultra-Lum UVC-515 Ultraviolet Multilinker at 1200 Joules/m².

2.5.3 Membrane hybridisation and blotting

a) Preparation of\textsuperscript{32}P-labelled probes

Labelling of DNA probes was carried out using DNA-gigaprime labelling kit (Geneworks). Briefly, 500ng-1µg DNA fragment was denatured at 95°C for 10 min and then placed on ice. Denatured DNA was immediately added to a mix containing 4µl each of dCTP, dTTP, dGTP, 5µl \(\alpha^{32}\text{P}\)-dATP (50µCi), 5µl reaction mix, 2 units Klenow and incubated at 37°C for 20 min. Labelled probes were purified through Bio-Gel P-6 column (BIORAD) and then denatured at 95°C for 10 min. 50µl denatured salmon sperm 'carrier' DNA was added to the probe, which was finally mixed with hybridisation solution and incubated with membrane overnight at 65°C.

b) Hybridisation and signal detection

Membranes were pre-hybridised at 65°C in glass bottles (HYBAID) in 10-15ml DNA hybridisation buffer (6xSSC, 0.2% SDS, 5x Denhardt's solution, 100µg/ml denatured salmon sperm DNA) for 3-5 h. Labelled probe was added to this and incubated with membranes overnight. Membranes were washed 3x 10 min in 2xSSC/0.1% SDS and 2-3 times for 15 min in 0.5xSSC/0.1% SDS (pre-heated to 65°C). Membranes exposed to X-OMAT film (KODAK) in autoradiograph cassettes with intensifying screens at -70°C and developed in an Ilford Ilfospeed 2240 X-ray processor.

2.6 Amplification and Sequencing of DNA

2.6.1 Primer design

The primers designed to dronc and decay are listed below:

Dr-F \(5'-GGAATTC\ ATGCAGCCGCGGAGC-3'\)
Dr-R \(5'-CGGGAATTC\ CTATTCGTTGAAAAACCCGG-3'\)
Primers designated with the letter ‘F’ are forward (sense) and the letter ‘R’ are reverse (anti-sense) primers, designed to the first ATG start and stop codon respectively. N-terminal or C-terminal tags are indicated in subscript. Mutated bases are underlined. Sequences in italics represent tags or restriction enzyme sites as follows:

Dr-FHA 5'-CCAAGCTTATGTCACCATACGCTCCAGACTACGTCGCAGCCCGAGGCTCAT-3'
Dr-RHSS 5'-CTAGTCTAGATTAATGATGATGATGATGATGTCGTTGGAAAAAAAAACCCCCGAT-3'
Dr-601 5'-AAGATACCCAGCGGTGCTC-3'
Dr-1015 5'-GCCGAAAAAGCAGCAAG-3'
Dr-RGFP 5'-CGGGATCCCTGTCGTTGGAAAAACCCCCGAT-3'
Dr-FGR5G 5'-ATGTTTCTCTTTGGCCCGCGGATG-3'
Dr-RGR5G 5'-CATCGCCCAGCGCGCCAAAGGAAACAT-3'
DrMPD-F 5'-GGAATTCCATATGGCAAGCCACCCCTTAATCTCG-3'
DrP14-F 5'-GGAATTCCATATGGTCGGCCATCAAGAATCAA-3'
DEC-F 5'-CCCAAGCTTATGCCCAAGGATCCGATA-3'
DEC-RFLAG 5'-CCGAATTTTCATACGTCCGTCGTTTTGAGTCGGTCTTGGTCAACGAG-3'
DEC-F(koz) 5'-GGCGGATCCCGCGCCATGGCCACCAAGATCCGATA-3'
DEC-RHSS 5'-CCGAATTTTCATACGTCCGTCGTTTTGAGTCGGTCTTGGTCAACGAG-3'
DEC-FGR5G 5'-CCGAATTTTCATACGTCCGTCGTTTTGAGTCGGTCTTGGTCAACGAG-3'
DEC-RGR5G 5'-CCGAATTTTCATACGTCCGTCGTTTTGAGTCGGTCTTGGTCAACGAG-3'
DEC-180 5'-TGCAAGCTCGCTCCTCCAT-3'
DEC-433 5'-CTTCATCCAGCGCTCCATGC-3'
DEC-RGFP 5'-GGCGGATCCCGCGCCATGGCCACCAAGATCCGATA-3'
DEC-FpET 5'-GCGGAATTCCATATGCCCCAGAAGATCCGATA-3'
DEC-783 5'-CTCAACCAGATGAAGAAAT-3'
T7 5'-GTAATACGACTCACTATAGGG-3'
Sp6 5'-GATTATCAGCTCACTATAG-3'
PRM-F 5'-AATGTGCACGTGTGAG-3'
PRM-R 5'-TTATTATCGGTATTAGGAAA-3'

Sequences in italics represent tags or restriction enzyme sites as follows:
GAATTC – EcoRI
GGATCC – BamHI
AAGCTT – HindIII
CATATG – NeoI
TCTAGA – XbaI

2.6.2 Primer purification

Oligonucleotide primers were synthesised at the IMVS/HCCR, Department of Tissue Pathology by Mr. A. Mangos and were purified by butanol extraction. 100μl deprotected oligonucleotide/ ammonium hydroxide solution was mixed with 1ml butanol and vortexed vigorously for 30 seconds. Oligonucleotides were pelleted by centrifugation at 9000g, 1 min. Following aspiration of the supernatant, DNA was air-dried and resuspended in 100μl sterile H₂O. Primer concentration was determined by spectrophotometry at absorbance of 260nm (OD₂₆₀ 1 = 20 μg/ml). Primers were also purchased commercially through Geneworks. In this case oligonucleotides were received as dried pellets and were resuspended to 100ng/μl in sterile H₂O.

2.6.3 Amplification of DNA by polymerase chain reaction

a) Taq polymerase amplification

Polymerase chain reaction (PCR) was carried out in a 50μl volume comprising DNA template (5μl first strand cDNA or 100ng plasmid DNA), 1x amplification buffer, 2.5mM MgCl₂, 100ng each of forward and reverse primers, 200μM of each deoxynucleotide-triphosphate (dNTP) (dATP, dTTP, dCTP, dGTP), 0.5 μl AmpliTaq polymerase (Perkin Elmer) in sterile H₂O. After addition of all components to 0.5ml PCR tubes (Treff), the reaction was overlaid with 50μl mineral oil and DNA synthesised in a DNA Thermal Cycler, (Hybaid) according to the required conditions. Standard reactions were denatured at 95°C for 1 min, then subject to 25-35 cycles of denaturation at 94°C 1 min, primer annealing at 50°C-55°C (depending on primer composition) 1 min, and primer extension at 72°C for 2-5 min (depending on the size of DNA fragment to be amplified,
generally 1 minute per kilobase). A final 10 min extension at 72°C was performed to ensure completion of DNA synthesis and reactions were held at 4°C.

b) Amplification using Pfu polymerase

Pfu is a high fidelity DNA polymerase (Stratagene) and was employed when amplifying DNA for cloning as it contained proof reading activity and therefore greatly reduced the risk of incorrect base incorporation. DNA was synthesised using 100ng DNA template, 200µM dNTP mix, 100ng forward and reverse primers, 100ng template DNA, 1x reaction buffer (20mM Tris-HCl pH 8.75, 10mM KCl, 10mM (NH₄)₂ SO₄, 2mM MgCl₂, 0.1% Triton X-100, 0.1mg/ml BSA) and 2.5 units Pfu polymerase in a total volume of 50µl sterile H₂O. Reactions were overlaid with mineral oil and synthesis carried out in a DNA thermal cycler (Hybaid). Amplification conditions consisted of an initial denaturation at 95°C for 1 min, followed by 25-30 cycles of denaturation at 95°C, primer annealing at 45-55°C (dependent on primer composition) for 1 min and primer extension at 72°C for 3-5 min. All reactions were held at 4°C.

c) Pwo polymerase amplification

Amplification of DNA was carried out using the high fidelity Pwo (Pyrococcus woesei) polymerase (Roche) once it became available. DNA was synthesised in a 50µl reaction comprising 100ng template DNA, 1x PCR buffer (10mM Tris-HCl pH 8.85, 25mM KCl, 5mM (NH₄)₂SO₄, 2mM MgSO₄), 2mM dNTP mix, 100ng each forward and reverse primers and 2.5 units Pwo polymerase in sterile H₂O. Reactions were overlaid with light mineral oil and amplified according to the required conditions. Manufacturer’s recommended amplification conditions were 1 cycle of denaturation at 94°C for 2 min, followed by 30-35 cycles of denaturation at 94°C for 30 sec, primer annealing at 50-55°C (depending on primer composition) for 30 sec and extension at 72°C for 45 seconds per kilobase of DNA to be synthesised. A final 72°C extension was carried out for 10 min and reactions were held at 4°C.
2.6.4 Site directed mutagenesis

Mutation of single DNA bases was carried out using the Quikchange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's protocol as follows: 20ng plasmid DNA template was amplified in a 50 µl reaction comprising 1x reaction buffer (10mM KCl, 10mM (NH₄)SO₄, 20mM Tris-HCl pH 8.8, 2mM MgSO₄, 0.1% Triton X-100, 0.1mg/ml nuclease-free BSA), 125ng of each mutagenic primer, 2mM dNTP mix, 2.5U *Pfu* DNA polymerase in sterile H₂O. Reactions were overlaid with sterile mineral oil and amplified by one cycle of denaturation at 95°C for 30 sec, followed by 12 cycles of denaturation at 95°C for 30 sec, primer binding at 55°C for 1 min and extension at 68°C for 2 min/kb of plasmid length. On completion of reaction, template DNA was digested by incubation with 1µl *DpnI* restriction enzyme in Buffer 4 (NEB) at 37°C for 90 min. Reaction product was checked on a 0.8% agarose/TAE gel and 5µl of this mutated DNA was transformed into chemically competent bacterial cells as described in section 2.2.2. Plasmid DNA was extracted and purified as described in section 2.3.2 and sequenced to confirm the presence of the desired mutation.

2.6.5 Sequencing

Plasmid DNA purified by BresaPure Midiprep protocol (section 2.3.2) or PCR fragments purified by BRESAclean protocol (section 2.1.4) were sequenced using the ABIPRISM Dye Terminator Cycle Sequencing Reaction Kit. 200-500ng template DNA was added to a sequencing reaction mix consisting of 100ng primer, 8µl Terminator Ready Reaction Mix (A/C/G/T-Dye Terminator, dGTP, dATP, dCTP, dTTP, Tris-HCl pH9.0, MgCl₂, thermal stable pyrophosphatase, AmpliTaq polymerase) and the volume was made up to 20 µl with sterile H₂O. The sequencing reaction was performed in a DNA thermal cycler (Hybaid) using the following conditions: [96°C -10 sec, 50°C -5 sec, 60°C -4 min] x 25 cycles, followed by a 4°C hold. DNA was precipitated by the addition of 2µl of 3M NaAc pH 4.6 and 50µl of 100% ethanol, on ice for 10 min. DNA was pelleted by centrifugation at 9000g, 15 min, 4°C, washed in 70% ethanol, air-dried and sequenced using a Perkin Elmer automated sequencer.
2.7 Generation of Plasmid DNA Constructs

2.7.1 Sub-Cloning vectors

a) pOT2 EST clones

pOT2-DRONC: Original vector supplied by Berkley Drosophila Genome Project. Contains full-length dronc cDNA (2.1kb) in the EcoRI / XhoI sites. Contains T7 and Sp6 promoter primer sites for sequencing dronc insert.

pOT2-mDRONC^{C318G}: DRONC cysteine mutant (C318G) was generated by Quikchange™ mutagenesis using pOT2-DRONC as template and Dr-F^{C318G/R^{C318G}} mutagenic primers.

pOT2-DECAY: Original vector supplied by Berkley containing full length decay cDNA (1.1kb). Also contains an extra ~700 base pair 3’sequence that appears to be derived from fusion of a heterologous cDNA unrelated to decay sequence. cDNA was sequenced using T7, Sp6 primers as well as some internal decay primers (section 2.6.5).

pOT2-mDECAY^{Cl50G}: DECAY cysteine mutant (C150G) generated by Quikchange™ mutagenesis using pOT2-DECAY as template DNA and DEC-F^{Cl50G/R^{Cl50G}} primers.

b) pBluescript constructs

pBs-DRONC: dronc cDNA was amplified by PCR using Pfu polymerase from pOT2 vector using 5’Dr-F/R primers, purified by phenol:CHCl₃ extracted and digested with EcoRI and ligated into EcoRI cut pBs (SK⁻).

pBs-mDRONC^{C318G}: DRONC cysteine mutant (C318G) was generated by Quikchange mutagenesis using pBs-DRONC as template and Dr-F^{C318G/R^{C318G}} mutagenic primers.
2.7.2 Mammalian expression constructs

**pcDNA3-DRONC/ mDRONC\(^{C318G}\):** DRONC (wild type and C318G mutant) coding sequence was released from pBs-DRONC/mDRONC\(^{C318G}\) respectively by HindIII/XbaI digestion and cloned directionally into pcDNA3 expression vector (Invitrogen). HA and/or His\(_6\)-tagged versions of DRONC and mDRONC\(^{C318G}\) were generated by PCR amplification using Dr-F\(_{HA}\)/Dr-R\(_{His6}\) primers from their respected pBs templates. PCR products were digested with HindIII/XbaI and directionally cloned into pcDNA3.

**pcDNA3-DRONC(MPD):** DRONC minus pro-domain (MPD) fragment was amplified by PCR with DrMPD-F/Dr-R primers. PCR product was digested with EcoRI and ligated into EcoRI/EcoRV pcDNA3.

**pGFP-DRONC/mDRONC\(^{C318G}\):** To generate constructs in which *Aequorea victoria* green fluorescent protein (GFP) was fused to DRONC, both wild type and the catalytic cysteine mutant of DRONC were amplified by PCR using Dr-F/ Dr-R\(_{GFP}\) primers, digested with EcoRI/BamHI and directionally cloned into pEFGFP-N1 (CLONTECH). This generated C-terminal GFP fusion proteins.

**pcDNA3-DECAY/ DECAY\(^{C150G}\):** DECAY (wild type and C150G mutant) cDNA was amplified by PCR using DEC-F\(_{Koz}\)/R\(_{FLAG}\) primers. DEC-F\(_{Koz}\) primer contained a consensus Kozak sequence that required alteration of the initiation site from the original sequence. DEC-R\(_{FLAG}\) primer contained the sequence encoding the FLAG tag. Amplified PCR product was digested with HindIII/EcoRI restriction enzymes and ligated directionally into pcDNA3.

**pcDNA3-P35:** Provided by Dr Vishva Dixit (Genentech, San Fransisco, USA)
pCXN2-CrmA: DNA fragment encoding CrmA (a kind gift from Dr David Pickup) was isolated as a 1.1kb EcoRI/HindIII fragment from pGEM7 vector, treated with T4 polymerase and cloned blunt ended into pCXN2 vector (Niwa et al., 1991).

pCXN2-OpIAP: cDNA for OpIAP was kindly provided by Dr David Vaux (Walter and Eliza Institute of Medical Research, Melbourne, Australia). OpIAP was released as a BamHI/XbaI fragment from pEFpuro vector, end-filled and cloned blunt into pCXN2.

pCXN2-MIHA: cDNA for MIHA was also provided by Dr David Vaux. MIHA was amplified by PCR using primers with terminal EcoRI sites and a 3' FLAG-tag sequence and cloned into the EcoRI site of pCXN2.

pRSV-Bcl2: This Bcl-2 expression construct was kindly provided by David Vaux.

pcDNA3-RPRFLAG, pcDNA3-GRIMFLAG, pcDNA3-HIDFLAG. Constructs kindly provided by Dr Vishva Dixit (Genentech, San Francisco, USA). Each encode a C-terminal FLAG-tag.

pcDNA3-DCP1HA: dcp-1 cDNA was amplified by PCR from pT7-dcp1 template (a gift from Dr Hirotaka Kanuka, RIKEN, Japan). Primers used were flanked with HindIII/XbaI restriction sites and a 3' HA-tag sequence. Amplified product was cloned directionally into pcDNA3 vector.

pcDNA3-DCP2HA: dcp-2 cDNA was amplified by PCR from pT7-DREDD plasmid (provided by Dr John Abrahams). Primers were flanked with EcoRI/XbaI sites and a 3' HA-tag sequence and PCR product was cloned directionally into pcDNA3.

pcDNA3-drICEHA: drice cDNA was amplified by PCR from pRSET-drICE (provided by Dr Masayuki Miura, RIKEN, Japan). Primers used for amplification contained
EcoRI/XbaI sites and a 3' HA-tag sequence. Product was cloned directionally into pcDNA3.

**pcDNA3-DIAP1MYC**: DIAP1 cDNA was released from pBs-DIAP1MYC (provided by Dr Bruce Hay) with EcoRI/BamHI and cloned directionally into pcDNA3.

**pcDNA3-DIAP2HA**: DIAP2 cDNA was amplified by PCR from pBs-DIAP2 to generate a 3'HA tag. Amplified product was cloned into the EcoRV site of pcDNA3.

### 2.7.3 Insect Expression Constructs

pRMHa3, pUAST and pGMR plasmids were provided by Dr Helena Richardson (Peter MacCallum Institute, Melbourne). Vectors are described in Brand and Perrimon (1993) and Richardson *et al.* (1995) (Figures 2.1 - 2.3)

**pRM-DRONC/mDRONC<sup>C318G</sup>/ DRONC(MPD)**: DRONC (wild type, C318G mutant and minus prodomain) fragments amplified by PCR and ligated blunt into pRMHa3.

**pRM-DECAY/mDECAY<sup>C150G</sup>/ DECAY(MPD)**: DECAY (wild type, C150G mutant and minus prodomain) inserts were released from their pcDNA3 vector constructs with BamHI/XhoI and cloned into the BamHI/SalI sites of pRMHa3 vector.

**pRM-DCP1<sub>HA</sub>/DCP2<sub>HA</sub>**: DCP1 and DCP2 cDNAs were amplified from their pT7 templates by PCR to generate a 3'HA tag fusion, and cloned blunt into pRMHa3.

**pRM-drICE<sub>HA</sub>**: drICE cDNA was amplified from pRSET-drICE by PCR to generate a 3'HA-tag fusion. Product was digested with EcoRI and cloned EcoRI/Blunt into pRMHa3.

**pRM-DIAP1<sub>HA</sub>**: DIAP1 cDNA was HA-tagged by PCR amplification from pBs-DIAP1MYC. PCR product was digested with EcoRI/BamHI and cloned directionally into pRMHa3.
Figure 2.1 pRMHa3 *Drosophila* expression vector. Expression of inserts are driven by the metallothionein promoter. The 3' end of Drosophila ADH gene and polyadenylation site follows the polylinker.
pRM-DIAP2 HA: DIAP2 was amplified from pBs-DIAP2. Primers used for PCR contained 5' EcoRI site and 3' HA-tag sequence. Amplified product was digested with EcoRI, and cloned EcoRI/Blunt into pRMHa3.

pRM-p35HA: p35 was amplified by PCR from pcDNA3-p35 plasmid to generate a 3'HA-tag fusion sequence. Amplified product was digested with EcoRI/BamHI and directionally cloned into pRMHa3.

pRM-RPRFLAG/GRIMFLAG: Reaper-FLAG and Grim-FLAG fragments were digested out of pcDNA3 with BamHI/XhoI and cloned into the BamHI/SalI sites of pRMHa3.

pRM-HIDFLAG: Hid-FLAG cDNA was released from pcDNA3 with HindIII/EcoRI and treated with Klenow to generate 'blunt' ends. This fragment was cloned blunt into pRMHa3 that had been digested with EcoRI and end-filled.

pRM-DARKMYC, pRM-DARK$_{1-411}$MYC, pRM-DARK$_{91-411}$MYC: Kindly provided by Dr John Abrahams.

pCaSpeR.h-lacZ: Supplied by Dr Masayuki Miura.

pUAST-DRONC/mDRONC$_{C318G}$: Wild type or C318G mutant DRONC fragments tagged with GFP were digested out of their pGFP constructs with NotI/EcoRI and cloned into the corresponding sites of pUAST.

pGMR-DECAY/mDECAY$_{C150G}$: Wild type and C150G mutant DECAY fragments were released from pRMHa3 vector with EcoRI and cloned into the EcoRI site of pGMR plasmid (adapted from the pCaSper-hs vector).
Figure 2.2 pUAST transgenic expression vector. pUAST is a transposable P-element vector and contains five yeast UAS, GAL4 binding sites. Target genes are cloned into a polylinker following the UAS promoter region and hsp70 TATA box. (adapted from Sullivan et al., 2000)
Figure 2.3 pGMR expression vector. Expression of inserts are driven by the *Drosophila* eye specific, glass minimal region (GMR) promoter.
2.7.4. *Bacterial expression constructs*

**pET32b-DRONC/mDRONC\(^{C318G}\):** Wild type, minus pro-domain (MPD) and C318G mutant DRONC were amplified by PCR using Dr-F or DrMPD-F and Dr-\(R_{GFP}\) primers. Fragments were digested with NdeI/BamHI and cloned directionally into PET32b vector (Novagen).

**pGEX4T1-DRONC/mDRONC\(^{C318G}\):** Wild type and C318G mutant DRONC were amplified by PCR with Dr-F/R primers, digested with EcoRI and cloned into the EcoRI site of pGEX4T1 plasmid (Amersham Pharmacia Biotech) to generate a 5’GST fusion.

**pGEX4T1-DRONC\(^{P14}\):** The small subunit (p14) of DRONC was amplified by PCR using 5’Dr-P14 and 3’Dr-R primers, digested with EcoRI and cloned into the EcoRI site of pGEX4T1.

**pET32b-DECAY/mDECAY\(^{C150G}\):** Wild type and C150G mutant DECAY were PCR amplified using DEC-F\(_{GFP}\)/DEC-R\(_{PET}\) primers. PCR product was digested with Ncol/BamHI and cloned directionally into pET32b.

**pRSET-DCP1/DCP2/DRICE:** Kindly supplied by Dr Masayuki Miura.

2.8 RNA Analysis

2.8.1. *RNA quantification*

RNA concentration was determined by measuring the absorption at 260nm on a spectrophotometer, assuming that O.D.\(_{260nm}\) of 1.0 represents 40\(\mu\)g/ml of RNA. Integrity of RNA was also checked by electrophoresis on 1% agarose/TAE gels and ethidium bromide stained bands were visualised under short wave-length UV.
**2.8.2 RNA extraction**

*a) Total RNA extraction*

Suspension cells were harvested, pelleted and resuspended in 2ml RNAzol™B (BIOTECHX LABORATORIES, INC.) per $10^7$ cells. *Drosophila* embryos, pupae or adult flies were homogenised in 500µl RNAzol™B per 20 flies. 100µl chloroform per 1ml homogenate was added, mixed vigorously for 15 sec and incubated on ice for 5 min. The suspension was then centrifuged at 9000g, 15 min, 4°C and the aqueous phase containing RNA was transferred to an RNase free microfuge tube. An equal volume of isopropanol was added to precipitate RNA and incubated on ice for 20 min. RNA was pelleted by centrifugation at 9000g for 25 min at 4°C, washed with 70% ethanol, air-dried and resuspended in 50-100µl diethylpyrocarbonate (DEPC) (Sigma) -treated sterile H$_2$O.

*b) Poly A* RNA purification*

PolyA* RNA was purified using Dynabeads® mRNA Purification kit (DYNAL). Prior to use 1ml (1mg) Oligo-(dT)$_{25}$ dynabeads was washed once in 500µl binding buffer (20mM Tris-HCl pH7.5, 1M LiCl, 2mM EDTA) and then resuspended in 500µl binding buffer. 100µg of total RNA was adjusted to a volume of 100µl in DEPC-treated water. RNA secondary structures were disrupted by heating samples to 65°C for 5 min and total RNA was mixed thoroughly with 100µl pre-washed oligo-(dT)$_{25}$ dynabeads. mRNA was annealed to the beads by gentle rotation for 30 min at room temperature. The tube was placed on the DYNAL MPC-E-1 magnet for 30 seconds. Beads were washed twice in 200µl Washing Buffer (10mM Tris-HCl pH7.5, 0.15M LiCl, 1mM EDTA) using the magnet to separate oligo-(dT)$_{25}$ beads from buffer ensuring that all wash buffer is removed in each step. mRNA was eluted from the beads by addition of 10µl Elution Buffer (10mM Tris-HCl pH7.5) and incubation at 65°C for 2-5 min. The tube was placed immediately onto the magnet and mRNA transferred to a clean microfuge tube and quantitated.
2.8.3 RNA gel electrophoresis

RNA samples were prepared as follows: 20µg total RNA or 2-5µg of PolyA+ selected RNA was mixed with formaldehyde running buffer (4µl formaldehyde, 10µl formamide, 2.5µl 10x MOPS solution, 1µl ethidium bromide [400µg/ml]). Samples were denatured at 65°C for 10 min, chilled on ice and mixed with 2µl RNA loading dye (50% glycerol, 1mM EDTA pH8, 0.25% bromophenol blue, 0.25% xyylene cyanol). 5µl RNA molecular weight markers (Roche) were denatured and treated the same way. Samples were loaded onto a 1.2% agarose gel containing 1x MOPS (20mM MOPS pH7, 1mM EDTA pH8, 8mM NaAc), 2.2M formaldehyde in DEPC treated water, and electrophoresed dry (with buffer just below the level of the gel) in 1xMOPS buffer. Gels were electrophoresed at 80 volts for 2 h, or until bromophenol blue dye front has run ¾ way through the gel, washed several times in sterile water and scanned using FluorImager 595 (Molecular Dynamics) with a 610nm filter.

2.8.4 Northern blotting

RNA from gels was transferred onto Biodyne transfer membrane (Pall) by capillary action overnight in 20x SSPE buffer (175.3g/L NaCl, 27.6g/L NaH₂PO₄, 7.4g/L EDTA pH7.4) as described for southern transfer (section 2.5.2). Following transfer, RNA was cross-linked in an Ultra-Lum UVC-515 Ultraviolet Multilinker at 1800 Joules/m². Membranes were pre-hybridised at 42°C for 4-5 h, in 10ml RNA hybridisation buffer (50% formamide, 5xSSPE, 1mM EDTA pH8, 5x Denhardt's solution, 0.1% SDS, 100µg/ml denatured salmon sperm DNA) in a HYBAID oven. Membranes were probed overnight with a DNA probe prepared using Bresatec DNA labelling kit as described in section 2.5.3 a). The following day, hybridisation buffer was discarded and membranes washed 3 times in 2xSSC/0.1% SDS for 10 min each followed by 2-3 washes in 0.5xSSC/0.1% SDS at 65°C. Membranes were exposed to X-ray film (KODAK) at -70°C over 1-5 days and films were processed using an Ilford Ilfospeed 2240 X-ray processor.
2.8.5 In situ mRNA analysis

An antisense digoxigenin-labelled riboprobe was prepared by transcribing from EcoRI linearised pOT2-dronc/decay cDNA using SP6 RNA polymerase. The sense riboprobe was prepared using T7 RNA polymerase from pOT2-dronc/decay cDNA linearised with XhoI. Digoxigenin labelling was according to manufacturer’s instructions (Roche). Briefly, 1µg linearised plasmid DNA was mixed with 2µl 10 x DIG RNA labelling mix, 2µl 10x transcription buffer, 2µl appropriate RNA polymerase in a total volume of 20µl. Reactions were incubated at 37°C for 2 h and stopped by the addition of 2µl 0.2M EDTA pH8 on ice. For in situ hybridisations, embryos and dissected larval tissue were fixed in 0.1M Hepes, 50mM EGTA, 0.01% Nonidet P40, 4% paraformaldehyde pH 6.9 for 20 min. Dissected ovaries from 3 day old adult females were fixed and treated with 50% ethanol/50% xylene for 30 min, washed in ethanol, then methanol and finally in PBS with 0.01% Triton X-100 (PBT). Ovaries were then refixed for 25 min in 4% paraformaldehyde and then treated with proteinaseK (5µg/ml) for 8 min at room temperature. Fixed sections were washed several times in PBS/0.05% Tween20 (PBST) and then in hybridisation buffer (50% deionized formamide, 5x SSC, 50µg/ml heparin, 100µg/ml denatured salmon sperm DNA, 0.1% Tween20) for 10 min each. Embryos and tissues sections were pre-hybridised in 400µl buffer, for 1 h at 55°C. Riboprobe was heat denatured and hybridised to sections overnight in 100µl hybridisation buffer. After hybridisation, non-specifically bound probe was removed by digestion with RnaseA (125µg/ml in PBST) for 1 h at 37°C. Hybridisation was detected using the secondary antibody detection system (Roche) as follows: Sections were blocked in 10% skim milk in TBST (100mM Tris HCl, 150mM NaCl pH7.5, 0.05% Tween20) for 1 h and then incubated with anti-DIG antibody (1:2000 in 10% milk/TBST) for 1 h at room temperature. Unbound antibody was washed 5 times for 20 min each in PBST, followed by 3x 20 min washes in AP buffer (100mM NaCl, 50mM MgCl2, 100mM Tris HCl pH9.5, 0.1% Tween20). Colour development substrates nitro blue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP) were mixed in AP buffer and added to embryo/tissue sections and stained for at least 1 h in the dark to allow colour to develop.
Reactions were stopped by rinsing several times in PBST/20mM EDTA and sections were mounted in 80% glycerol.

2.8.6 RNA interference (RNAi)

dronc and decay sense and anti-sense RNA transcripts were synthesised using the Ambion Megascript kit, using linearised pcDNA3-dronc/decay as template. Briefly, linearised plasmid DNA was purified by Bresaclean (section 2.1.4). 1µg linearised plasmid DNA was used in a transcription reaction with 10x reaction buffer, 75mM dNTPs (for T7 transcription) or 50mM dNTPs (Sp6 transcription) and 2.5 units enzyme mix in a total volume of 25µl at 37°C for 3 h. Template DNA was degraded by incubation with 1 unit Rnase-free DnaseI at 37°C for 15 min. RNA was purified by phenol chloroform extraction, followed by ethanol precipitation. Transcripts were dissolved in injection buffer (0.1mM NaPO₄ pH 7.8, 5mM KCl) at 0.75mg/ml and annealed by heating to 85°C and cooling to room temperature. All annealed transcripts were analysed on agarose gels with DNA markers and quantitated. 0-40 minute old embryos were dechorionated and aligned. Precellularized embryos were injected at 50% egg length and aged until stage 11-13 and then processed for immunostaining.

2.9 Protein Analysis

2.9.1 Protein concentration determination

Protein concentration was determined by Bicinchoninate (BCA) as per manufacturers protocol (PIERCE). Briefly, several dilutions of purified protein or lysates were prepared, along with serial dilutions of the protein standard, bovine serum albumin (BSA) ranging from 1mg/ml-0.125mg/ml. Reagents A and B were mixed together in a ratio of 50:1 and 100µl of this mix was incubated with 100µl of each diluted protein sample at 37°C for 30 min. The optical density at 562 nm for each protein sample was measured and a standard curve, from the BSA readings, constructed. Protein concentration was calculated from the standard curve.
2.9.2 Preparation of protein extracts

*Drosophila* whole fly protein lysates were prepared as follows: Wild type (w^118\textsuperscript{C}D\textsuperscript{4}), *dark^C* larvae, pupae and adult flies were frozen in liquid nitrogen and homogenised using a mortar and pestle. Approximately 10-20 lysed flies were resuspended in 300-500μl caspase assay buffer (0.1M Hepes pH7, 0.1% CHAPS, 10% PEG, 10mM dithiothreitol, supplemented with protease inhibitor cocktail tablet [Roche]). SL2 cell lysate was prepared by freeze thawing 1x10\textsuperscript{7} cells in 500μl caspase assay buffer. Lysates were centrifuged at 13000rpm for 10 min at 4°C, and supernatant were removed and centrifuged again for 10 min. The clear lysate was removed and used immediately or stored at -20°C. Whole cell protein extracts were prepared by resuspending cells in PBS with an equal volume of 2x protein loading buffer [PLB](100mM Tris-HCl pH 6.8, 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and boiled for 10 min prior to loading samples on a gel.

2.9.3 Expression of recombinant protein in E.coli

Overnight cultures of *Escherichia coli* BL21 (DE3) harbouring the pGEX4T1-DRONC (full length and p14 subunit), pET32b-DRONC or pET32b-DECAY expression plasmids were subcultured 1/50 in 200ml luria broth, grown for 3 h at 37°C, induced with 0.5mM-1mM isopropyl β-D-thiogalactoside (IPTG) and grown for an additional 3 h at 37°C. Culture was pelleted at 800g for 15 min and bacterial cell pellets were resuspended in phosphate buffered saline (PBS), lysed by sonication and clarified by centrifugation at 10,000g for 10 min at 4°C. GST-fused proteins were further purified by addition of 300μl pre-washed Glutathione Sepharose (Amersham Pharmacia Biotech) to the lysate and incubation for 2 h at 4°C. The sepharose was washed three times in PBS and bound DRONC-GST/P14-GST fusion proteins were eluted by incubating with 300μl glutathione elution buffer (50mM Tris-HCl pH 8, 10mM reduced glutathione) five times for 15 min each.
2.9.4 In vitro translation

cDNAs were transcribed and translated in vitro using the Promega TNT™ Coupled Reticulocyte Lysate System. In a standard reaction, 35S-Methionine labelled protein was translated in a 50µl reaction volume comprising 25µl TNT Rabbit Reticulocyte Lysate, 2µl TNT Reaction Buffer, 1µl T7 or SP6 polymerase, 1µl amino acid mixture (minus methionine), 4µl 35S-methionine, 1µl RNasin Ribonuclease inhibitor, 1µg DNA template and sterile H2O. Reactions were incubated at 30°C for 90 min and were used immediately or stored at -20°C for no more than one week.

2.9.5 Proteolysis assays

5µl of 35S-Met labelled protein was incubated in proteolysis assays at 37°C for 3 h in the presence of either cytoplasmic extracts, purified proteases, bacterially expressed caspases or with 100µg protein extracts prepared from whole flies. Assays were incubated in a total volume of 20µl in caspase assay buffer. Where indicated, inhibitors were used at the following concentrations: 1µM YVAD-fluoromethylketone (fmk) (Bachem), 1µM DEVD-CHO (Bachem) or 1µM VAD-fmk (Bachem). Inhibitors were pre-incubated with cell extracts, for 30 min at 37°C prior to the addition of 35S-Met labelled in vitro translated protein. Where indicated, 2µg cytochrome c and 2mM dATP were added to reaction. Reactions were terminated by the addition of an equal volume of 2x PLB. Samples were boiled for 5 min, centrifuged at 9000g for 5 min and cleavage products resolved by SDS polyacrylamide gel electrophoresis. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (NEN Life Science Products) membrane using semi-dry apparatus (Biometra) and visualised by autoradiography.

2.9.6 Fluorogenic caspase activity assays

A total of 100µg cleared protein lysates or 20µg GST-purified protein was assayed for caspase activity by incubation with 100µM of either DEVD-7-amino-4-methylcoumaride (DEVD-amc) (Enzyme System Products), VDVAD-amc (California Peptide Research), VEID-amc, LEHD-amc or IETD-amc (Bachem/Calbiochem) in a total volume of 40µl in caspase assay buffer at 37°C for 30 min. Reactions were transferred to acryl cuvettes.
(Sarstedt) and 3ml H₂O was added. Fluorescence was quantified on a Luminescence Spectrometer (Perkin-Elmer) (excitation 385nm; emission 460nm).

2.9.7 Immunoprecipitation Assays

a) Direct protein binding assays

Full length P35-HA, GRIM-FLAG, DIAP1-MYC, drICE-HA and DRONC were translated in vitro from pcDNA3 template using TNT T7 coupled Reticulolysate Lysate System (Promega). P35-HA, GRIM-FLAG and drICE-HA were purified by immunoprecipitating overnight at 4°C in a total volume of 400μl caspase assay buffer. Proteins were pulled down with protein G-sepharose (Amersham Pharmacia Biotech) and unbound protein washed off with wash buffer (50mM Tris-HCL pH 7, 150mM KCl, 2mM dithiothreitol, 0.025% Triton-X 100). 5μl P35-HA, GRIM-FLAG or drICE-HA, immobilised on protein G-sepharose, was added to purified DRONC-GST or 3⁵S-DRONC and / or 3⁵S-DIAP1 in a total volume of 400μl caspase assay buffer. After incubation at 4°C for 3h, beads were washed twice with 100 volumes of wash buffer and twice in 100 volumes of phosphate buffered saline before SDS-PAGE and immunoblot assay.

b) In vivo protein immunoprecipitation assay

5x10⁵ 293T cells or 3x10⁶ SL2 cells were seeded into 6cm dishes in 2 ml medium. The following day cells were co-transfected with DNA plasmids of interest (as described in section 2.11.4 below). Cells were harvested 24 h later, by rinsing once with PBS on ice and lysed in 1.5ml lysis buffer A (50mM Tris-HCl pH 7.6, 150mM NaCl, 0.1% NP40) or lysis buffer B (20mM Tris-HCl pH 7.4, 135mM NaCl, 0.2%-0.5% TritonX-100, 10% glycerol), supplemented with Complete™ protease inhibitor cocktail and 200μM NaVO3, 1mM NaF, 500μM Na pyrophosphate (Roche) for 30 min on ice. Cells were scraped and transferred to a 1.5ml microfuge tube and debris was removed by centrifugation at 9000g for 10 min at 4°C. Lysate was transferred to a clean microfuge tube and pre-cleared with 20μl protein-G-Sepharose for 2 h at 4°C with rotation. Sepharose was pelleted at 9000g for 2 min and lysate transferred to a clean tube. Proteins were immunoprecipitated overnight with 2-4μg of the appropriate antibody at 4°C with rotation. 20μl Protein-G-
Sepharose was added and incubated a further 2 h at 4°C to allow antibodies to bind. Antibody/Sepharose complex was pelleted at 9000g for 2 min and washed twice in lysis buffer followed by two washes in PBS. 20μl 2x protein loading buffer was added before SDS-PAGE and immunoblotting.

2.9.8 SDS-PAGE and protein transfer

Polyacrylamide gels of the appropriate percentage (10-15% polyacrylamide [BIORAD], 37.5mM Tris pH8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.05% TEMED[GIBCO]) were cast and layered with 5% stacking gel (5% polyacrylamide, 0.125M Tris-HCl pH6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED). Gels were assembled into large vertical protein electrophoresis tanks (Owl Scientific) or Hoefer minigel tank apparatus (Amersham Pharmacia Biotech) and immersed in protein electrophoresis buffer (25mM Tris, 250mM glycine, 0.1% SDS). Samples were mixed with an equal volume of 2 x protein loading dye (100mM Tris-HCl pH 6.8, 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min. Debris was pelleted at 9000g for 5 min and samples loaded and electrophoresed at 100 volts through the stacking gel and then 200 volts through the resolving gel. Following electrophoresis, protein was transferred to a sheet of polyvinylidene difluoride (PVDF) (“Polyscreen” Dupont) membrane using a Hoefer semi-dry transfer apparatus (Amersham Pharmacia Biotech). For protein transfer, the gel, PVDF membrane and 4 sheets of Whatman filter paper, cut to the exact size of the gel, were all pre-soaked in protein transfer buffer (49mM Tris, 39mM glycine, 0.0375% SDS, 20% methanol). Protein transfer was set up with 2 sheets of Whatman placed on the cathode (+), and then stacked with the PVDF membrane, followed by the gel and the final 2 sheets of Whatman. Protein was transferred at 130 mAmp for 1.5h.

2.9.9 Coomassie staining

To visualise proteins, gels were stained with Coomassie Brilliant Blue R-250 (Biorad) (0.25% w/v in H₂O:MeOH:acetic acid, 5:4:1). Gels were stained for at least 1 h, followed by destaining in several changes of H₂O:MeOH:acetic acid (5:4:1) for 1-2 h. Gels were dried between cellophane sheets (BIORAD) under vacuum at 80°C for 30 min.

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2.9.10 Immunoblotting

Following protein transfer PVDF protein filters were blocked in 5% skim milk (Diploma) PBS-T solution for 1 h at room temperature or overnight at 4°C. Primary antibodies were diluted in 5% skim milk PBS-T solution as follows; DRONC affinity purified rabbit antibody 1/300 for 4 h, GFP and c-MYC mouse monoclonal and α-HA rat monoclonal antibodies (Roche) diluted 1/1000 for 2 h, FLAG mouse monoclonal antibody (KODAK) diluted 1/1000 for 2 h. Following blotting with primary antibody, filters were washed in PBS-T; 2x 5 min and then 2x 10 min. Secondary antibodies used were either anti-rabbit-IgG conjugated with horseradish peroxidase (HRP) (Amersham Corp.) to detect rabbit polyclonal antisera, anti-mouse-IgG or anti-rat-IgG conjugated with HRP (Amersham Pharmacia Biotech) to detect mouse and rat monoclonal antibodies respectively. Secondary antibodies were diluted 1/2000 in 5% skim milk /PBS-T solution and were incubated for 1 h at room temperature. Following secondary antibody incubation, filters were washed in PBS-T as described above, and signals were visualised by enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Equal volumes of the two ECL reagents were mixed and poured onto a glass plate. The membrane was placed protein side down onto the ECL solution mix and left for 1 min after which it was wrapped in clear plastic film and exposed to Hyperfilm ECL (Amersham-Pharmacia). Exposure times ranged for 5 sec to 1 h depending on the intensity of signal required.

2.9.11 Immunoblot stripping

To reblot protein membranes with other antibodies, they were first washed 2 x 5 min in PBS-T and then antibodies were stripped by incubation in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) for 5 min at 60°C. Membranes were then washed 2x 10 min in PBS-T and blocked in 5% skim milk PBS-T for 1 h at room temperature prior to blotting with primary antibody.
2.10 Antibody Production

2.10.1 Protein purification

GST-DRONC fusion protein was produced as described in section 2.9.3. The carboxyl-terminal (-P14) fragment of DRONC was purified from 4x 200ml cultures. Protein concentration was determined by BCA assay as described in section 2.9.1.

2.10.2 Protein inoculation

DRONC P14 antigen was used to inoculate two rabbits. Rabbits were inoculated with antigen over a 12 week period; 400µg P14 was primarily inoculated, followed by 3 booster shots of 200µg antigen every 3 weeks. Pre-immune serum from rabbits following all booster immunisations was tested for its affinity on a western blot as described in section 2.9.10.

2.10.3 Antibody affinity purification

Polyclonal antibody to DRONC was purified through a DRONC (P14) antigen coupled-Sepharose column. 5mg of DRONC GST-P14 protein was incubated with the sepharose overnight at 4°C. The sepharose/P14-coupled complex was washed once in coupling buffer and incubated for 2 h at room temperature with 0.1M Tris-HCl, 0.5M NaCl, pH 8.0. The Sepharose was then washed in 0.1M Na Acetate, 0.5M NaCl, pH 4.0 followed by 4 washes in coupling buffer. The Sepharose was then packed into a column and washed with 0.1M phosphate buffer (0.1M Na₂PO₄, Na₂HPO₄, pH 8.2). 1.5ml of serum from the rabbits immunised with DRONC GST-P14 protein was diluted in an equal volume of 0.1M phosphate buffer and passed through the column. The column was washed 5 times with phosphates buffer. The affinity purified antibody was eluted by the addition of 0.2M glycine, pH 3.0 to the column and 0.5ml fractions collected into 0.5ml of 1M Tris-HCl, pH 8.0/ 0.1% w/v azide. The absorbance of each fraction was measured at wavelength of 280nm and protein concentration measured using the following formula; [protein] mg/ml = OD₂₈₀ x 0.74. Fractions containing affinity purified antibody were pooled and the antibody dialysed overnight at 4°C against PBS/0.1% w/v azide. 1ml aliquots of antibody were stored at -20°C in the presence of 50% glycerol. The antibody
was tested for its affinity on a western blot as described in section 2.9.10. A 1/300 dilution was found to be optimal to detect transfected DRONC protein.

2.11 Tissue Culture

2.11.1 Cell lines and culture conditions

All cell culture was carried out in Class 2 'biohazard' laminar flow hoods (Gelman Sciences). The adherent human embryonic kidney 293T (HEK-293T) cell line was grown in RPMI 1640 (GibcoBRL). The adherent murine fibroblast NIH-3T3 cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) (GibcoBRL). Media contained 2% sodium hydrogen carbonate, 10 % HEPES, 1% penicillin (Glaxo), 1% streptomycin sulphate (Glaxo), and pH adjusted to 7.4 (RPMI) or pH 7 (DMEM) with 1M HCl. All media was further supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamine and pre-warmed to 37°C prior to use. Adherent cells were harvested with trypsin. Cells were washed with phosphate-buffered-saline (PBS) and treated in trypsin (0.054%w/v trypsin [Difco], 0.54 mM EDTA in Hank’s Balanced Salt Solution [HBSS]) for 1 minute to detach cells from the flask surface and to obtain a single cell suspension. Medium containing 10% FBS was added to inactivate trypsin and cell density and viability assessed by trypan blue (0.8% w/v in PBS) exclusion using a haemocytometer. Cells were maintained at density 5x10^5 cells/ml in 100mm diameter sterile tissue culture dishes, at greater than 95% viability. All mammalian cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

Schneider L2 (SL2) cells were maintained in Schneider Cell Medium (GibcoBRL) supplemented with 10% fetal bovine serum (GibcoBRL), 1% glutamine, 0.5% penicillin/streptomycin/neomycin mix (GibcoBRL) and incubated at 27°C. Cell medium was warmed to room temperature prior to use. SL2 cells were harvested by washing once in medium and scraping cells off using a cell scraper (Costar). Cell density and viability determined as above and cells maintained at density of 5x10^5 cells/ml in a 75cm² flask (Greiner-Cellstar). Cell viability was maintained at 90-95%.
2.11.2 Cryopreservation of cells

Cells were harvested and resuspended at 5-10 x 10^6 cells/ml in medium/10% FBS. To mammalian cell suspension, an equal volume of cryoprotectant freezing mix (30% heat-inactivated FBS, 20% dimethyl sulphoxide [DMSO] (BDH, Merck), 50% RPMI 1640) was added slowly to cells. To SL2 cells an equal volume of freezing mix (20% FBS, 10% DMSO, 70% Schneider cell medium) was added. Cell suspensions were dispensed into 1ml aliquots in 1ml cryotubes (Nunc), and cells frozen by controlled rate freezing and stored in liquid nitrogen filled tanks.

2.11.3 Thawing cryopreserved cells

Once removed from liquid nitrogen, mammalian cells were thawed rapidly at 37°C and added slowly to 5ml pre-warmed medium. Cells were washed twice in 10ml medium by centrifugation at 2500rpm for 5 min each. Cells were then seeded into an appropriately sized tissue culture flask in medium. SL2 cells were thawed at room temperature and seeded directly into a 25cm² flask. Cells were allowed to adhere to flask for 1 h at 27°C after which cells were washed twice in Schneider cell medium and incubated in 5ml fresh medium at 27°C.

2.11.4 Transient transfection assays

a) Transfection of mammalian cells with FuGENE

Mammalian cell transfections were carried out using FuGENE6 reagent. The day before transfection, NIH-3T3 cells or 293T cells were seeded at densities of 2.5x10^5 cells per well into 6 well tissue culture plates or at 6x10^5 cells into 60mm dishes, in 2ml and 3ml medium respectively. The following day, cells were co-transfected with 1.5µg plasmid DNA expression constructs, contained in the pCXN2 vector (Niwa et al., 1991), and 0.5µg of the β-galactosidase expression vector (pEF-βgal) (Kumar et al., 1994). Where indicated pCXN2-DRONC or pCXN2-DECAY was co-transfected with pCXN2-CrmA, pCXN2-P35, pCXN2-MIHA, pCXN2-OpIAP or pRSV-Bcl2 expression constructs at a ratio of 1:2. Transfections were carried out using FuGENE6 reagent (Roche). For small-scale transfections in 35mm dishes, 6µl FuGENE6 reagent was diluted into 100µl in
serum and antibiotic-free medium, and incubated for 5 min at room temperature. For larger scale transfections in 60mm dishes, 4µg DNA was mixed with 12µl FuGENE6 pre-diluted in 150µl serum free medium. The diluted FuGENE 6 reagent was then added drop-wise to 2µg plasmid DNA and incubated for 15 min at room temperature. The Fugene / DNA mix was added drop-wise to cells with gentle mixing, into medium containing serum and incubated overnight at 37°C. At 18-24 h after transfection, cells were analysed for β-galactosidase expression by fixing in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min, washing twice with PBS and then staining with 1mg/ml X-gal, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM MgCl₂ in PBS, for 3 h at 37°C. β-gal positive blue cells were analysed microscopically for apoptotic morphology and photographed. The extent of apoptosis was represented as % morphologically apoptotic cells among the total number of positive (blue) stained transfected cells.

b) Transfection of SL2 cells using Cellfectin reagent

For cell death assays using SL2 cells, 2x10⁶ cells were seeded into 60mm dishes in 2ml medium the day before transfection. Cells were co-transfected with 1.5µg metallothionein-inducible pRMHa3 vector containing DRONC (pMT-DRONC -wild type, C318G mutant, minus pro-domain [MPD]) or DECAY (pMT-DECAY -wild type, C150G mutant) along with 0.5µg of the heat shock inducible pCasper.hulacZ reporter plasmid. Where indicated plasmid DNA was co-transfected with pMT-p35, DIAP1, DIAP2 or DRONC³ⁱ₈ expression constructs at a ratio of 1:2. Transfections were carried out using Cellfectin reagent (GibcoBRL) as follows: In two separate tubes, 2µg total DNA was diluted to 100µl and 9µl Cellfectin reagent diluted to 100µl in Schneider serum free Sf-900II media (SFM-GibcoBRL). Diluted Cellfectin reagent was added dropwise to diluted DNA and incubated for 20 min at room temperature after which 800µl SFM was added. Cell monolayers were washed once with 2ml SFM and incubated with the 1ml DNA/lipid complex at 27°C for 4 h, after which media was aspirated and cells fed with 2ml Schneider cell medium containing serum. 16 h after transfection, cells were heat-shocked at 37°C for 30 min and then allowed 30 min recovery time at 27°C. This was repeated another two
Following heat shock, cells were split into halves in two 35mm dishes, one dish treated with 0.7 mM CuSO₄. Where indicated, 50μM z-VAD-fmk (Enzyme Systems Inc) was added to cells at the time of CuSO₄ addition. 48 h after CuSO₄ induction, cells were stained for β-galactosidase and % surviving cells calculated as the % β-gal positive cells in CuSO₄ treated cells relative to % β-gal positive cells in untreated dishes.

2.11.5 SL2 death assays

SL2 cells were transiently transfected with either DRONC or DECAY using Cellfectin as described above (section 2.11.4b). Expression of DRONC or DECAY was induced with CuSO₄ for 16 h, before treatment with etoposide (Calbiochem) at 40μM, UV irradiation (50-100 Joules/m²) or cycloheximide (Sigma) at 25μg/ml. Cells were induced with each stimuli over a 24 h period and harvested at 0, 2, 4, 8, 16 and 24 h.

2.11.6 Immunofluorescence assays

Cells were seeded onto ethanol sterilised cover slips, in 6 well dishes (Costar) and left to sit overnight at 37°C for mammalian cell lines and 27°C for SL2 cells. Cells were transfected with DNA as described in section 2.11.4 and left overnight at the appropriate temperature. Following transfection SL2 cells were CuSO₄ treated overnight prior to fixation and staining. For immunostaining, cells were washed in PBS and fixed in 1ml methanol: acetone: formaldehyde mix (47.5%: 47.5%: 5%) for 30 min at room temperature. Cells were washed three times in 2ml PBS to remove fixative and then incubated in the presence of primary antibody anti-FLAG or anti-HA at 1:200 dilution in PBS / 1% FCS for 30 min at room temperature. Antibody was then washed off with three changes of PBS and cells were stained with secondary antibody (anti mouse IgG-FITC / anti rat-FITC or anti-mouse IgG-rhodamine) diluted 1:200 in PBS/1% FCS for 30 min. Secondary antibody was washed off with three changes of PBS and coverslip was mounted onto a slide with a drop of 80% glycerol. Cells were viewed under fluorescence microscope with the appropriate filters to detect either FITC or rhodamine staining.
2.12 *Drosophila melanogaster* manipulation

For all fly manipulations, refer to Sullivan *et al.*, (2000)

2.12.1 *Fly maintenance*

Fly stocks were maintained in vials containing a yeast food mix: 10g/L agar, 100g/L polenta, 186g/L yeast, 143g /L treacle, 25ml/L tegosept (100g methyl parahydroxybenzoate, 2.5g/L tetracycline in 1L ethanol) and 15 ml/L acid mix (orthophosphoric acid: propionic acid at 1:10 ratio in water). All flies were stored and aged at 18°C. Genetic interaction studies were carried out at 18°C, 25°C or 29°C to regulate gene dosage, according to toxicity of expressed gene

2.12.2 *Embryo collection*

Crosses were set up in lay tubes which was placed over a grape agar plate (35g/L J Grade agar, 20g/L sucrose, 30ml tegosept) containing a globule of yeast. Flies were left to lay eggs onto the agar plate at room temperature. Resulting embryos were flushed off the plate with PBS-T and brushed into microfuge tubes. For staging embryos, eggs were collected at set time points and aged as time after egg laying. Collecting larvae and pupae was carried out by directly separating larvae from adult fly stocks. To distinguish the two, larvae are lighter in colour and pupae have developed mandibula and eyes begin to pigment during later pupae stages.

2.12.3 *Sexing*

Collection of adult flies was carried out under CO₂ and flies viewed under a light microscope. Generally, females are larger with a pointed abdomen. Males can be distinguished by their rounded abdomens, and black pigmentation of posterior tergites. Males can easily be distinguished by their genitalia as they have torsal sex combs.

2.12.4 *Virgin collection*

Virgins were separated from adult flies under light microscopy. Virgin females are very pale and dark meconium in the abdomen is visible.
2.12.5 Dechorionation and fixation of embryos

Embryos were aspirated in 50% bleach / 50% PBS-T for 2 min then washed thoroughly with PBS-T. Embryos were placed in fixative solution (0.5x Buffer A [1M HEPES, 0.5M EGTA, 0.1% NP40], 2% formaldehyde, 50% heptane) for 20 min at room temperature, with agitation. Bottom layer of solution was removed and replaced with an equal volume of methanol to remove the vitelline membrane. Embryos were shaken until they sunk to the bottom, this is an indication that the vitelline membrane has been removed. Embryos were washed with three changes of 1 ml methanol for 5 min each, at room temperature. To rehydrate embryos, half the solution volume was replaced with 50% methanol / 50% PBS-T and shaken for 5 min. 1ml of PBS-T was added and shaken a further 5 min, followed by several washes in PBS-T. Embryos can be used directly for staining and *in situ* or can be stored in ethanol and kept at 4°C. If storage was required, embryos were rehydrated prior to use.

2.12.6 Microinjecting embryos

Embryos were dechorionated and dessicated essentially as described above. Embryos affixed to coverslip and aligned in the same orientation and in parallel. Coverslip was then affixed to a slide with oil. To dessicate, slide was placed in a silica-gel containing box for 10 min after which eggs were covered immediately with oil. To inject, mounted embryos were pierced at their posterior pole with a needle and injected with DNA or double stranded RNA, until embryo was full but before any leakage. After injections, eggs were left on the slide under oil at 18°C until they hatched. Larvae was then removed from the oil into fly food or onto grape agar plates containing yeast, resulting larvae or adult flies further manipulated for *in situ* hybridisations or genetic interaction studies.

2.12.7 Transgenic fly generation

To direct gene expression in *Drosophila*, transgenic lines were generated by microinjecting *pUAST-droncGFP* or *pUAST-droncC186GFP* constructs under the control of the *GAL4/UAS* promoter. Once the target gene randomly integrated into the genome,
the chromosomal location of the inserted gene was determined by crossing flies to specific balancer chromosome fly stocks, which have distinct phenotypes. The target gene is kept silent in the absence of GAL4. To activate the gene of interest in a tissue specific manner, flies carrying the target gene are crossed to flies expressing GAL4 (Enhancer Trap GAL4) in the specific tissue of interest. In our case, transgenic UAS-dronc or UAS-dronc\textsuperscript{C318G} flies were crossed to the eye specific enhancer GMR-GAL4, such that GAL4 expression is restricted to the fly eye, and the eye phenotype analysed. Many of the UAS-dronc lines were lethal at 25°C, whereas other lines gave only a few adult survivors with severe eye defects. None of the UAS-dronc\textsuperscript{C318G} lines resulted in lethality. Two less severe lines, UAS-dronc\#80 and UAS-dronc\#23, both contained a double insertion of the transgene on the second and third chromosome, and were used for genetic analysis. For both of these lines, recombinants were generated containing UAS-dronc and GMR-GAL4 (second chromosome) and were made homozygous for the UAS-dronc insert on the third chromosome.

2.12.8 Fly crosses and genetic interaction studies

The UAS-dronc/GMR-GAL4 expressing lines were crossed at 25°C or 29°C to wild type (\textit{w}1118) flies; to GMR-p35, GMR-diap1, GMR-diap2 expressing flies; to strains containing a deficiency of \textit{rpr}, \textit{hid} and \textit{grim} [\textit{Df}(3L)H99], \textit{diap1} [\textit{Df}(3L)brm11 and \textit{Df}(3L)stf-13], or \textit{diap2} [\textit{Df}(2R)Jp1]; to a specific loss of function allele of \textit{diap1 [thread]}; or crossed to P allele mutations of \textit{dark} or \textit{dark\textsuperscript{CD4}}, \textit{dark\textsuperscript{CD8}} and \textit{l(2)k11502} hypomorphic alleles. To test the interaction of a dronc deficiency \textit{Df}(3L)\textit{ACI} at 67A2-67D13 with GMR-\textit{hid} or GMR-\textit{rpr}, crosses were carried out at 25°C and 18°C respectively. Progeny were scored by examining the eye phenotypes using light or scanning electron microscopes. Deficiency GMR-p35 (third chromosome) and \textit{l(2)k11502} stocks were obtained from the Bloomington Stock Center.

2.12.9 Immunohistochemistry

Antibody staining of \textit{Drosophila} tissues were performed as follows: Embryos were aged and dechorionated, dehydrated and rehydrated as described in section 2.12.5.
Embryos were then blocked in 10% milk / PBS-T for 1 h at room temperature and then rinsed in PBS-T. Primary antibody, α-DRONC, α-GFP or Mab 22C10 was added at a 1:200 dilution and gently shaken overnight at 4°C. Embryos were washed in three changes of PBS-T over 20 min. Secondary antibody, anti-rabbit rhodamine or α-mouse FITC respectively, was added at a 1:200 dilution in 10% milk / PBS-T and incubated for 1 h at room temperature. Embryos were once again washed with three changes of PBS-T for 5 and then 20 min each, and then mounted in 80% glycerol in PBS-T and viewed under a fluorescence microscope. The 22C10 antibody was developed by S. Benzer (Department of Biology, Caltech, Pasadena, California) and obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The anti-GFP rabbit antibody was obtained from P. Silver (Department of Cell Biology, Harvard Medical School, Boston, MA).

2.12.10 Detection of apoptotic cells in vivo

Embryos were dechorionated prior to stainings. Tissue sections were dissected from embryos or larvae in PBS or Schneider Cell medium (GIBCO-BRL).

a) TUNEL staining

Embryos were fixed in 4% paraformaldehyde for 30 min, divitellinized in 80% ethanol and rehydrated in PBS. Embryos washed in PBS-T followed by a wash in 1x terminal transferase buffer (2.5mM CoCl2, 0.3% TritonX-100) (Roche). Embryos were incubated for 3 h at 37°C in 100μl reaction buffer (1x terminal transferase buffer, 0.5U/μl terminal transferase, 10μM dUTP consisting of a 1:2 mix of biotin-16-dUTP and UTP) (Roche). Biotinylated nucleotides were visualised with fluorescein isothiocyanate (FITC)-avidin at a 1:200 dilution in PBS-T for 1 h. Reaction was stopped by washing in PBS-T and embryos or tissue sections were mounted in 80% glycerol and visualised under confocal microscopy.
b) Hoechst staining

Hoechst (10mg/ml) was diluted 1:1000 in PBS-T and embryos shaken in this solution for 5 min at room temperature. Embryos rinsed with three changes of PBS-T over 20 min. PBS-T was replaced with 80% glycerol and flies stored at 4°C until viewed under fluorescence microscopy.

2.12.11 Ecdysone treatment of larval salivary glands and midgut

Larvae midgut and salivary gland were dissected in Schneider cell medium and then incubated in 500μl of 1 mM 20-hydroxyecdysone (Sigma) in Schneider cell medium for 1 h at 25°C. Tissues were fixed in 4% paraformaldehyde / 1x Buffer A and in situ hybridisations with an antisense, digoxigenin-labelled dronc or decay riboprobes were performed as described in section 2.8.5.
Chapter 3

Identification of DRONC, an ecdysone-inducible Drosophila caspase
3.1 Introduction

Caspases are essential mediators of programmed cell death (PCD) in metazoans. While *C. elegans* contains three caspases, only CED-3 is essential for all developmental cell deaths (Ellis and Horvitz 1986; Horvitz *et al.*, 1994). There are currently 14 mammalian caspases and it is becoming apparent that although some caspases have compensatory functions, other caspases function in a tissue specific manner and may regulate specific pathways to death during development (Zheng *et al.*, 2000).

As a simplified model system of apoptosis, *Drosophila melanogaster* has been manipulated to further our understanding on the regulation of caspases. At the commencement of this project, only three *Drosophila* caspases had been identified, DCP-2/DREDD and drICE. Four *Drosophila* caspases have since been cloned in our laboratory, two of these are named DAMM and STRICA (Doumanis *et al.*, 2001; Harvey *et al.*, 2001) and the other two are described in the following chapters. DREDD and STRICA have been classified as Class I apical caspases based on the presence of an amino terminal prodomain. Although STRICA does not appear to contain any protein interaction motifs, DREDD contains a DID that mediates interaction with dFADD (Hu and Yang, 2000). To date, it is unclear whether DREDD, like Caspase-8, acts in a death receptor signalling pathway or, more importantly whether a Fas-like death pathway exists in the fly. The remaining *Drosophila* caspases act as downstream, Class II caspases, and cleave various cellular proteins in the execution of apoptosis.

The aim of this study was to identify other upstream initiator caspases to further our knowledge of cell death regulation in the fly. This chapter describes the identification of a novel Class I *Drosophila* caspase, DRONC, which contains an amino-terminal caspase recruitment domain (CARD). Results in this chapter demonstrate that *dronc* expression is dramatically upregulated in the salivary gland and midgut by ecdysone, which is the first indication that a caspase functions in steroid-mediated apoptosis during insect metamorphosis. Furthermore, DRONC is processed and activated in response to various apoptotic stimuli and by various caspases. Results presented in this chapter have been published (Dorstyn *et al*. 1999; Harvey *et al.*, 2001).
Results

3.2 Identification of DRONC as a unique caspase

There are nine mammalian caspases that contain an amino-terminal CARD, essential for oligomerization and autoactivation of caspases and/or caspase recruitment to upstream adaptor proteins. To identify CARD-containing proteins in Drosophila, the prodomain of Nedd2 (mouse Caspase-2) was used to search the Berkeley Drosophila genome database in a TBLASTN program. A Drosophila expressed sequence tag (EST) was identified, which contained a small open reading frame sharing 29% sequence identity with the Caspase-2 prodomain over an 88 amino acid stretch. This EST is part of a cluster of 11 ESTs obtained from the Berkley Fly Database. The sequence of two independent cDNA clones revealed the presence of a complete open reading frame encoding a novel caspase that we named DRONC (Drosophila Nedd-2 like caspase). The full-length dronc cDNA sequence of 2160 nucleotides has been deposited in Genbank (accession number AF104357). The genomic sequence of dronc comprises a 5' untranslated region (UTR) and a 1351 bp coding sequence that is divided into two exons by a 510 bp intron (Figure 3.1A). dronc has been mapped to chromosome 3 region 67C4-C8, a region that is not well characterised. In the annotated Drosophila genome, the coding region of dronc is on the (-) strand. The complete mRNA coding sequence is displayed in Figure 3.1B.

The putative DRONC protein consists of 450 amino acid residues (Figure 3.2A). In vitro translation of mRNA generated from dronc cDNA produced a 50 kDa protein consistent with the expected size (Figure 3.2B). A unique feature of DRONC is its PFCRG active site, encompassing the catalytic cysteine (Cys318) residue, which is distinct from the conserved QACRG sequence found in the majority of other known caspases. Full length DRONC shares 25% identity (40% similarity) with Caspase-2. The region downstream of the prodomain, which encodes the large and small sub-units of DRONC, is highly homologous to Caspases-3, -7, -8, -9, -10 and CED-3, sharing approximately 28% identity and between 44-48% similarity. DRONC shares only 24% identity (38-44% similarity) with DCP-1 DCP-2/DREDD, drICE and DAMM but does not show significant similarity to STRICA. Interestingly, of all the Drosophila caspases, DRONC is most similar to DECAy, sharing 27% identity and 45% similarity (described in chapter 5).
Figure 3.1 Genomic structure and sequence of *dronc*.

(A) Genomic structure of the *dronc* gene at 67C4-C8, displaying exon-intron organisation. Position of the start and termination codons are indicated, with coding regions in blue and non-coding regions shown as open boxes. Scale bar is shown on the top right.

(B) Sequence of *dronc* cDNA including 5' and 3' untranslated regions (UTR). The full-length nucleotide sequence of *dronc* cDNA comprises 2158 bp with the open reading frame extending from base 370 to 1722 (in bold). The intron insertion site is indicated with a red arrow.
A

5' UTR  exon 1  exon 2  3' UTR

1  ttggccgcg  cgaaagctc  ttggtcttt  ttgtttgcga  agaaatccta  ttggagatc
61  cccogatcct  gttagagata  tctatgcga  tatataagct  aatgtaggtta  ctttttgaga
121  aataaggaca  ccagcagggc  gcgcgatttg  tcgccccttc  ttgaaagtgc  caaaaacaaa
181  acaacagca  caaacaacaa  ggagagacaa  aagaatgaca  agtagcagata  aacgaatatac
241  ttccccggga  aacccttgag  aaccgtgatt  caatgcaaat  accaactgca  ttgtagagact
301  gatcaagggca  gccatctcttg  ggctgccccaa  taagcggagct  cagcggagcg  aatgagaccc
361  atatccggga  tgcaagccgacct  gtagctcag  cgaagagggca  tgcgagcgcg  taggcgcgat
421  ataogcaaga  atctgtagat  aagttgctga  ttggccgaact  aagagcgtct  ggcogcatgag
481  tcgtcgcaac  aacgctcctt  atcgctcag  atctgaagaa  aacagcgagt  ctcctctccc
541  aagcaatcca  atccagacg  gaagagacgg  cgtatctgag  tcgtctatgt  gctcctatgg
601  aataagctgg  atgccggcat  cccctgcttc  gcctgtccttc  tccgctgcac  ctcctctgtc
661  aatgtagcgg  ttctatgtag  ttgctgctctg  agtctcagtc  aacggcaacc  aagctctgcct
721  ttatctctga  taaaagcaac  gagaaccagc  cgtttcaggt  ccggatcttt  gcctcctccc
781  tcccgccgga  aacccgttgc  aaccgtgcttt  gcaagtgcac  gctcctctcc  gcctgtccttc
841  cctatccctg  atgtagcggtt  cttatgctgtt  cggagttcgg  aagctctgcct  gccgtctctc
901  gttggggata  gtgcctattg  ggcactgat  aagataacat  cggcttccttc  gcgttgtgc
961  ttgctaatgg  ttacatacatg  gcactatcctg  gtcacaaaccc  gttgacctgg  ggagcgccgaa
1021  aagagcaagc  agtcgctgtat  acatctgttc  caaagactga  aatattacat  tttgcctctc
t81  ggagaagctga  atcagggacta  gttcttttttta  cttcttaca  cgtttggtta  tctctcttttt
1141  gtgcacaaaga  ctggatgctttg  cttaactgctg  ctgtagctgca  agccgcaagc  tgtgctgagga
1201  aaagagatgg  tgtggatgctt  gatgtgctgt  ttgctgctgt  tgcagagatc  caagaggccat
1261  ttgacgacg  ccaaatgtcc  ttatgtggtg  aacaagccga  agggcttgtt  gttctctcct
1321  tgcctcggag  ctagctgctg  ctagctgctg  cttgggtgca  cccaaagatct  ggttctgagga
1381  gtctatggg  ggagctgcggt  gacaccagcgc  cggagggcact  cacccgagcgg
1441  agccacatag  tgcagctgtg  tgcacactgt  ttgattctgt  atctaaatgc  gccggctgat
1501  gttaaacaccc  gcctctcggc  tggcagatcc  cttgctatcc  caggttttcgt  ccaagtaactc
1561  gcctacatgc  ccacagcacc  agaacctgag  gatatemataa  aagacagcag  cagagcgcctg
1621  gtaaatagcg  gcaccaagga  ggttcctcatg  cgcacaggtg  cctctgataaa  tttgcctttt
1681  aataaaaaac  tcatactccaa  tcccggggtctt  ttgctaacgat  agtgcgccg  acgtacagct
1741  ttatcattcc  ggatgccattt  ttcacgccat  tttgtggcatt  atcgtgctgct  ttagattggc
t801  ttttagattta  tgggctgtgct  gtcgtgcgtgct  tataaatagtt  ttatatgtagta  caacataactc
1861  attcactatgg  tttctactaa  cgttagctct  acctcatttta  aagttgaaagttatcgtat
1921  ttatgtgata  acgtgagagg  atctctaaaa  attataacat  agagttcccag  tatataacca
1981  gcctcttcgg  tgaatagcagaa  cattgacaata  caattttcctca  gttctgtatct
2041  ttggtgctga  cactttccaa  ataaaagctga  atatcttcca  acaagacccca  gttgtatatat
2101  aatttaaatcc  tatattcatttt  tcatccttttt  cattgggaca  ctaattatta  tataatat

B

1  gtagaaatcctta  ttggtcttt  ttgtttgcga  agaaatccta  ttggagatc
61  cccogatcct  gttagagata  tctatgcga  tatataagct  aatgtaggtta  ctttttgaga
121  aataaggaca  ccagcagggc  gcgcgatttg  tcgccccttc  ttgaaagtgc  caaaaacaaa
181  acaacagca  caaacaacaa  ggagagacaa  aagaatgaca  agtagcagata  aacgaatatac
241  ttccccggga  aacccttgag  aaccgtgatt  caatgcaaat  accaactgca  ttgtagagact
301  gatcaagggca  gccatctcttg  ggctgccccaa  taagcggagct  cagcggagcg  aatgagaccc
361  atatccggga  tgcaagccgacct  gtagctcag  cgaagagggca  tgcgagcgcg  taggcgcgat
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1741  ttatcattcc  ggatgccattt  ttcacgccat  tttgtggcatt  atcgtgctgct  ttagattggc
Figure 3.2 Primary structure of DRONC protein.

(A) Deduced amino acid sequence of DRONC. The full-length protein comprises 450 amino acids. The active site residues are shown in bold, with the catalytic cysteine residue highlighted in red. (B) *In vitro* translation of DRONC shows that protein is 50 kDa in size. (C) Schematic representation of DRONC protein structure. The large and small subunits are predicted based on the potential cleavage sites (D113, D135, D324 and E352) in the DRONC precursor. The position of the pentapeptide sequence PFCRG containing the catalytic cysteine residue is shown as a hatched box. (D) Alignment of the CARD motifs of various caspases. The locations of the six α-helices (H1-H6) are marked. Residues conserved in at least four proteins are shaded in blue, those conserved in three proteins are shaded in green and those showing conservative changes are shown in grey. The DRONC CARD contains an extended linker region between H3 and H4, not present in other CARDS.
A

MQPPELEIGM PKRHERHIRK NNLNIVENTN YBRLAMECVQ QGILTQVQMLR NTQDLNGKPF 60
NMDEKDVVER QHRRLLLKIT QRGPTAYNLL INALRNINCL DAAVLLESVD ESDSRPFPIE 120
LNERRTSRKS ADIVDPYSEP ASEGPCVSKL RNEPLGALTQ YGVVDGPEV KKSKKINGGD 180
SAILOTYKMQ SRFNRGBVLM VNIMYDPQDN RRRIGAERDS KSLIHLPQEL NPTIPYPGVN 240
NQDQFPKTML MTVSSSYVQN TECRVMVLMT HGNSVEGKRR VBPFGDGESVYD MQIKHDHPQ 300
AKCPYLVNPQ KVLMEPPFCG DRYDLHPRKN QGVLNMEPVT AQEEKMDIQQ TGDIDPSTBN 360
VPSLADTLCVD YANTFCYVTH RDLDGWSYI QKFCQWMADH ARSDDLEDIL KKTSEAVGHNK 420
RTKGSQMTG AYDNGLFNNK LYPNPGPFFNE 450

B

50 kDa

C

D135
D113
D324

CARD
p24
p14

D

DRONC
CED-3
CASPASE-1
CASPASE-2
CASPASE-9

H1
H2
H3
H4
H5
H6

DRONC
CED-3
CASPASE-1
CASPASE-2
CASPASE-9

FNMDEKDVVER QHRLPLLLK KTQDPTAYNLL INALRNINCLDAAVLLES 108
VREKREIVKAVQORGDVA FDAYFDALBSTGHEGALEVE 90
VMKTRALDSVIPKGAQACICITYICE.EDSTLAGTGL 90
SFRSNVELLNLKSPCODASMCEALRETQGHifdefMLLT 120
SRRDCAOLILIDLETREGSOALPLFSCQEDTGQDMGSFIR 90
amino-terminal prodomain of DRONC encodes a CARD (Figure 3.2C) that comprises all six α-helices conserved in the CARD containing molecules CED-3, Caspases-1, -2 and -9 (Figure 3.2D). Putative aspartate residues that may be targets for DRONC processing to release 24 kDa and 14 kDa sub-units are illustrated in Figure 3.2C.

3.3 Expression of dronc during Drosophila development

Northern blot analysis of dronc mRNA detected a 2.2 kb transcript, consistent with the expected size of cDNA (Figure 3.3A). The transcript for dronc can be detected at both early and late embryonic stages and is substantially downregulated during first and early third instar larvae stages. Interestingly, late larvae / early pupae stages demonstrated upregulation of dronc, which was barely detectable in late pupae. To assess expression of dronc in adult flies, RT-PCR was carried out, using primers that amplified the entire dronc open reading frame. As displayed in Figure 3.3B, expression of dronc was also detected during adulthood in both male and female flies.

Consistent with Northern data and RT-PCR, in situ hybridisation analysis of dronc mRNA demonstrated high expression in stage 1-4 syncitial embryos (Figure 3.4). Zygotic expression does not begin before stage 5, so this early expression of dronc represents maternally derived mRNA. dronc mRNA is ubiquitously expressed in stage 8 cellularised embryos and decreases as development proceeds (Figure 3.4B and C). Unlike expression of dredd, dronc does not increase in apoptotic cells in embryos (Figures 3.4A-C).

Analysis of dronc expression in various second and third instar larval tissues demonstrated high level of dronc mRNA expression in midgut and salivary glands from late third instar larvae (4E-H) but not in second instar larvae (Figure 3.4I and J). Cell death in the midgut begins late during second instar larvae and becomes prominent at the onset of pupariation. In contrast, apoptosis in the salivary gland tissue begins 13.5 h after pupariation. It therefore appears that the high expression of dronc transcript precedes apoptosis of these tissues. In third instar larval eye discs and brain lobes, where many cells are undergoing apoptosis, dronc expression is low (Figure 3.4 K and M). The only staining detected appears to be contained within dying blood cells, which are often associated with imaginal discs (Figure 3.4K). Expression of dronc in ovaries is strong
Figure 3.3 Expression of dronc mRNA during Drosophila development.

(A) A Northern blot of total RNA from various developmental stages. dronc transcript is detected as a single 2.2 kb band. The lower panel shows a portion of the ethidium bromide stained gel with ribosomal RNA bands, before transfer to membrane, to indicate loading intensities of RNA. (B) RT-PCR analysis of dronc expression using dronc-specific primers that generated a 1.35kb product. Lower panel shows RT-PCR amplification of Drosophila ribosomal protein 49 (rp49). rp49 is expressed ubiquitously throughout development and therefore used as an internal control.
3.4 In situ analysis of dronc mRNA expression during Drosophila development.

Expression of dronc was detected by in situ hybridisation with a digoxigenin-labelled antisense mRNA probe.

(A) Stage 3 syncitial embryo showing high levels of dronc expression.

(B) Stage 8 embryo showing uniform dronc expression throughout.

(C) Stage 13 syncitial embryo with reduced dronc expression. Arrow indicates amniosera.

(D) Stage 13 embryo hybridised with a dronc sense control probe shows no background staining.

(E) Late third instar larval midgut with high dronc expression.

(F) Late third instar midgut hybridised with a control dronc sense riboprobe.

(G) Late third instar larval salivary gland showing high dronc expression.

(H) dronc sense control probe on late third instar larval salivary gland.

(I, J) Late second instar midgut and salivary gland respectively, showing low or no dronc expression.

(K) Late third instar eye imaginal disc shows ubiquitous low levels of dronc. Arrow shows higher level of staining in a subset of blood cells associated with the eye disc.

(L) dronc sense control probe on eye imaginal disc.

(M) Brain lobes from late second instar larvae showing ubiquitous low levels of dronc mRNA.

(N) Control dronc sense riboprobe on brain lobes.

(O) Adult egg chambers showing high expression of dronc particularly at the later stages of oogenesis. The stages of oogenesis for each chamber are indicated.

(P) dronc sense riboprobe on adult egg chambers.
within egg chambers predominantly after stage 10 (Figure 3.4O and P), suggesting that *dronc* expression precedes apoptosis of nurse cells during oogenesis (Buszczak and Cooley, 2000).

### 3.4 *dronc* mRNA expression is induced by ecdysone.

The high expression of *dronc* mRNA during early pupae stages in the midgut and salivary gland prompted the idea that DRONC may be a mediator of apoptosis of these tissues during metamorphosis. The steroid hormone ecdysone has been shown to be crucial for these apoptotic events (Baehrecke 2000). During larvae and pupae development there are large peaks of ecdysone activity that coincide with tissue remodelling and apoptosis (Figure 3.5B). To assess whether upregulation of *dronc* mRNA coincided with ecdysone activity, the various stages of pupae development were analysed for *dronc* expression. Northern blot analysis of the timing of *dronc* upregulation during pupae stages detected transcript between 12 and 16 h following pupation (Figure 3.5A).

To examine whether ecdysone induces *dronc* expression, second instar larval salivary gland and midgut, which express very low levels of *dronc* (Figure 3.4I and J) were treated with ecdysone (Figure 3.6). After a 1 h exposure to ecdysone there was a several fold increase in *dronc* mRNA levels in early second instar larval midgut, indicating that ecdysone induces *dronc* expression in midgut (Figure 3.6A). In contrast, salivary glands from early second instar larvae did not display *dronc* induction by ecdysone (data not shown). Salivary glands undergo apoptosis later than midgut so the lack of ecdysone-induced upregulation of *dronc* may have been due to the absence of a developmentally controlled factor required for ecdysone-induced gene expression at this stage. Consistent with this, salivary glands from a later second instar larval stage, which normally express very low levels of *dronc*, displayed strong *dronc* expression 1 h after ecdysone treatment (Figure 3.6A). Northern blot analysis of ecdysone-induced *dronc* up-regulation is demonstrated in Figure 3.6B. Quantitation of ecdysone-induced *dronc* mRNA levels has shown a >20 fold increase in the level of transcript in midgut and salivary glands. Expression of *dronc* in early pupae is shown as a comparison. Therefore ecdysone is able to induce *dronc* expression in both midgut and salivary gland tissues.
Figure 3.5 Expression of *dronc* during pupae development.

(A) Northern blot analysis of *dronc* mRNA expression at different pupae stages. Time is given as h after pupariation. Lower panel depicts ribosomal bands on the ethidium bromide stained gel prior to transfer. (B) Graph depicting waves of ecdysone activity during the different stages of *Drosophila* development. (adapted from Bate and Martinez Arias, 1993). Note the two peaks of ecdysone activity following pupariation, corresponds to *dronc* up-regulation.
### A

**Table:**

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</table>

**Legend:**

- **2.2 kb rRNA**
- **rRNA**

### B

**Graph:**

- **Ecdysteroid (pg/mg)**
- **Time (hrs):**
  - embry | 1st | 2nd | 3rd | pupa | adult
  - 0     | 24  | 48  | 72  | 96   | 120 |

**Key Events:**

- hatch
- ecdysis
- ecdysis
- wandering
- pupariation
- pupation
- eclosion

**Markers:**

- male
- fed female
Figure 3.6. *dronc* mRNA is up-regulated by ecdysone.

(A) An early second instar midgut and late second instar salivary gland, incubated for 1 h without (-) or with (+) ecdysone. Note massive induction of *dronc* expression upon ecdysone treatment in both midgut and salivary gland. The sense control riboprobe did not show any staining on tissues with or without ecdysone treatment. (B) Total RNA was prepared from untreated and ecdysone-treated tissues was subjected to Northern blot analysis using a *dronc* cDNA probe. The lower panel depicts a portion of the ethidium bromide stained gel before transfer to membrane. The last lane in the gel contains RNA from early pupae, which express relatively high levels of *dronc* transcript.
A  

<table>
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<td>Salivary Gland</td>
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B  

<table>
<thead>
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<th>Ecdysone</th>
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<th>Late 2nd Instar Salivary Gland</th>
<th>Pupae</th>
</tr>
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<tbody>
<tr>
<td>Time (h)</td>
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2.2 kb >
rRNA >
3.5 Ectopic expression of DRONC in cell culture induces apoptosis

Class I caspases are commonly characterised by their ability to induce apoptosis when overexpressed in various cell types (Kumar and Colussi 1999). To test whether DRONC has this ability in vitro, the murine fibroblast cell line NIH-3T3 and the mammalian human embryonic kidney cell line 293T, were co-transfected with DRONC and a β-galactosidase reporter vector. The morphology of β-gal positive, apoptotic NIH-3T3 cells, displaying membrane blebbing and cytoplasmic condensation induced by DRONC expression at 48 h, is shown in Figure 3.74-D. Interestingly, DRONC was unable to induce cell death in 293T cells but 48 h after transfection of NIH-3T3 cells, approximately 60% of the β-gal positive cells had undergone apoptosis (Figure 3.84). When co-expressed with various caspase inhibitors, DRONC-induced apoptosis was prevented by P35 and to a lesser extent by XIAP, OpIAP and Bcl-2. In contrast, CrmA was least effective at protecting cells from DRONC-induced death. A substitution mutant of the catalytic cysteine to a glycine residue (C318G) completely abrogated the cell killing activity of DRONC, suggesting that cysteine protease activity is responsible for the apoptotic function of DRONC.

To establish whether DRONC is also able to also induce apoptosis in Drosophila cells, DRONC was transfected into the hemocyte-derived Drosophila Schneider (SL2) cell line. In these transient transfection experiments, DRONC expression is induced by CuSO₄, and due to the fact that SL2 cells lift off rapidly when dying, percentage cell survival was quantitated by comparing the number of remaining β-gal positive cells in CuSO₄ induced versus uninduced samples (see Chapter 2, section 2.11.5). Surprisingly, full-length DRONC was only able to induce minimal (25%) cell death (Figure 3.8B). Previous studies have demonstrated that the prodomain region can have an inhibitory effect on caspase activity. For example, DCP-1 and drICE have substantially greater activity once their short prodomains have been removed (Fraser and Evan 1997). In addition, several caspase inhibitors, such as IAPs, are able to bind caspases via this amino-terminal region and consequently prevent their processing and activation (Hay 2000). The prodomain of DRONC was therefore removed and the remaining truncated protein, DRONC(MPD) [aa114-450], was transfected into SL2 cells. Removal of the
Figure 3.7 β-galactosidase positive cells demonstrating the morphological changes induced by DRONC expression.

NIH-3T3 cells were transiently transfected with pcDNA3 vector (A), pcDNA3-DRONC<sup>C<sub>318G</sub></sup> (B), or pcDNA3-DRONC (C, D) expression vectors, together with a pEF-β-galactosidase reporter vector, and stained with X-gal at 24 h (A-C) or 48 h (D) after transfection. Arrows depict cells transfected with pcDNA3-DRONC exhibiting a rounded, condensed morphology following 48 h.
Figure 3.8 DRONC induces apoptosis in transfected cells.

(A) NIH-3T3 cells were co-transfected with various expression constructs and pEF-β-gal reporter vector by lipofection. 48 h after transfection, cells were fixed and stained with X-gal, and blue cells were observed for apoptosis. (B) Drosophila SL2 cells were co-transfected with various pRMHa3 vector expression constructs as labelled, and pCasper-lacz. 24 h after transfection cells were heat shocked to induce lacZ expression and then treated with CuSO₄ (0.7 mM) for 48 h to induce DRONC expression. z-VAD-fmk (50μM) was added at the time of CuSO₄ addition. Cell survival was quantified by comparing the percentage of β-gal positive cells in CuSO₄ treated versus untreated dishes. In both (A) and (B), bars represent apoptotic cells as a percentage of total β-gal positive cells ± standard error of the mean (SEM). At least 400 blue cells were scored for each dish. The data shown were derived from three independent experiments.
prodomain significantly enhanced the level of apoptosis (50% at 48 h post-transfection) (Figure 3.8B). Interestingly DIAP1, which inhibits caspase activity though binding to their prodomain, was still able to prevent DRONC(MPD) induced death, suggesting that DIAP1 may interact with other regions of this caspase, or acts by inhibiting downstream caspases. In contrast, DIAP2 was unable to efficiently rescue cells from DRONC(MPD) mediated death. P35 was still able to afford an inhibitory affect, but not as well as z-VAD-fmk or DIAP1. The catalytically inactive DRONC^{C318G} mutant also significantly suppressed DRONC(MPD) activity in SL2 cells suggesting that it is able to act as a dominant negative mutant.

3.6 Localisation of ectopically expressed DRONC

The localisation of DRONC was analysed by transfecting cells with a GFP-DRONC fusion construct. The fusion of GFP to the carboxyl-terminus of DRONC did not effect its killing ability in NIH-3T3 cells (Figure 3.9A-D). At 24 h post-transfection, most of the cells appeared morphologically normal and DRONC or DRONC^{C318G} appeared to be localised in the cytoplasm of cells (Figure 3.9B and C). In some cells, DRONC was concentrated near the nucleus, possibly associated with some subcellular structures. Further analysis of DRONC localisation by staining of transfected cells with mitochondrial markers indicated that DRONC does not localise to mitochondria (data not shown). At 48 h after transfection, DRONC-GFP protein was uniformly distributed in apoptotic cells (Figure 3.9D). Analysis of DRONC-HA localisation in Drosophila SL2 cells revealed a similar distribution pattern when stained with α-HA antibody (Figure 3.9E-H). DRONC appeared to be mainly in cytoplasm, with some staining seen in the nucleus of high expressing cells (Figure 3.9G). Analysis of the localisation of DRONC(MPD), also demonstrated cytoplasmic staining but no nuclear staining was seen (Figure 3.9H).
Figure 3.9 Cellular localisation of DRONC.

NIH-3T3 cells (A-D) or SL2 cells (E-H) were transfected with various DRONC expression constructs and protein localisation was analysed by fluorescence microscopy. DRONC-GFP expression in NIH-3T3 cells was analysed at either 24 h or 48 h after transfection. DRONC expression in SL2 cells was analysed 24 h after CuSO₄ induction by immunostaining with α-DRONC antibody followed by α-rabbit-FITC conjugated antibody.

(A) pGFP vector at 24 h.
(B) pGFP-DRONC<sup>C318G</sup> at 24 h.
(C) pGFP-DRONC at 24 h
(D) pGFP-DRONC at 48h
(E) pRMHa3 vector
(F) pRMHa3-DRONC<sup>C318G</sup>
(G) pRMHa3-DRONC
(H) pRMHa3-DRONC(MPD)
3.7 Enzymatic activity and substrate specificity of DRONC

To confirm that DRONC is a caspase, recombinant DRONC was generated in Escherichia coli and assessed for its proteolytic activity on synthetic fluorogenic peptide substrates. Expression of both the full length DRONC precursor or DRONC(MPD) both generated active enzyme that showed low level activity on the Caspase-3 substrate DEVD-afc (Figure 3.10). In contrast, DRONC activity on the Caspase-2 pentapeptide substrate VDVAD-amc was 5 fold higher than activity on DEVD-afc. This finding suggests that, similar to Caspase-2, the optimal minimum substrate requirement for DRONC may include a P5 residue. No significant cleavage of the Caspase-1 substrate YVAD-afc by DRONC was observed (Figure 3.10).

3.8 Processing and activation of DRONC

To analyse the processing of DRONC by other caspases, in vitro translated and 35S-labelled DRONC protein was incubated with several Drosophila lysates or with active bacterial lysates expressing recombinant caspases. As displayed in Figure 3.11A, DRONC was efficiently processed by drICE into subunits of approximately 18kDa and 14kDa, indicating that although drICE is considered a Class II caspase, it is able to process Class I caspases in a possible amplification loop of apoptosis. DRONC could also partially process itself suggesting that it may, although inefficiently, auto-activate itself. An apoptotic SL2 cell extract was able to induce DRONC processing to a 36 kDa intermediate and 18 kDa and 14 kDa subunits bands can also be detected. Cleavage of DRONC by DCP-1 or DCP-2/DREDD could not be detected in our in vitro system, and it is not known whether these caspases can process DRONC in vivo. Cleavage of DRONC was also detected in the presence of active recombinant mammalian caspases (-3, -6 and -7) (data not shown), suggesting that DRONC can be processed at DEDD or VEID sites by these caspases. Extracts prepared from second and third instar larvae, but not from pupae, efficiently processed in vitro translated DRONC, to 32 kDa, 18 kDa and 14 kDa fragments, indicating the presence of caspase-like activity able to activate DRONC during larval stages of development (Figure 3.11A, second panel).
Figure 3.10 Activity of recombinant DRONC on fluorogenic peptide substrates. 

_E. coli_ lysates expressing recombinant DRONC, Caspase-2 or Caspase-3 were incubated with 100μM various fluorogenic caspase substrates, DEVD-amc, VDVAD-amc or YVAD-amc at 37°C for 30 min, and release of amc was monitored on a fluorimeter. Equivalent amount of _E. coli_ lysates lacking caspase expression (buffer) was used in control experiments. Bars represent average fluorescence units ± SEM derived from three independent experiments.
Figure 3.11 Processing of DRONC by active recombinant caspases and *Drosophila* lysates.

(A) $^{35}$S-DRONC processing by *Drosophila* caspases, SL2 cell extract or larval extracts. *In vitro* translated, $^{35}$S-labelled DRONC protein was incubated with the indicated caspases or lysates for 3 h at 37°C and processing analysed by autoradiography.

(B) DRONC bacterial lysate was pre-incubated with 1μM DEVD-CHO, YVAD-cmk or VAD-fmk for 30 min at 37°C, prior to the addition of $^{35}$S-DRONC and then incubated a further 3 h at 37°C. DRONC protein was detected by autoradiography.

(C) Schematic representation of DRONC cleavage after indicated aspartate residues to generate products of sizes 38 kDa, 36 kDa, 32 kDa and 14 kDa as seen in all cases. The 18 kDa and 9 kDa cleavage products seen must be generated by cleavage at alternative sites. The glutamate (E352) cleavage site is described by Hawkins and colleagues (2000).
C

B

A

14 / 12 KDa
24/22 KDa
28 KDa
12 KDa
38 / 36 KDa
50 KDa

DRONC

+VAD-fmk
+YVAD-fmk
+DEVD-fmk

DRONC

pupae
3rd instar
SL2

2nd instar
DAMM
DCP-2
DCP-1
DRONC

14
18
38
50 KDa
To further investigate the protease activity responsible for DRONC cleavage, DRONC was incubated with several caspase inhibitor peptides (Figure 3.11B). Self-cleavage of DRONC to generate a 38 kDa fragment, could be inhibited by the general caspase inhibitor VAD-fmk and partially by DEVD-cho but not by the Caspase-1 inhibitor YVAD-fmk (Figure 3.10). These results indicate that DRONC is unlikely to self-process at YVAD or DEVD sites.

A schematic representation of putative DRONC sites that generate the cleavage products seen is shown in Figure 3.11C. Interestingly, a 24 kDa cleavage product equivalent to the large subunit, could not be detected in any of the above in vitro assays. Instead an 18kDa band was detected, implying there may be further processing of this subunit at alternative sites. Recently a publication by Hawkins and colleagues (2000) demonstrated self-cleavage of DRONC at glutamate residue (E352). This is the first indication of alternative substrate specificity of a caspase and indicates that DRONC may process other caspases, in addition to itself, after glutamate residues.

To analyse the processing of Drosophila caspases by each other, in vitro translated DCP-1, DCP-2 or drICE were incubated with bacterial lysates expressing either recombinant DCP-1, DCP-2, drICE or DRONC. As shown in Figure 3.12, processing of DCP-1 was seen only with drICE. DCP-2 processing by Drosophila caspases was not detected whereas drICE was cleaved very efficiently by itself and partially by DRONC. These results suggest drICE may act as the downstream caspase substrate for DRONC.

3.9 DRONC is processed in response to apoptotic stimuli

To examine the processing and activation of DRONC during apoptosis, cell extracts were prepared from etoposide, cycloheximide and UV treated SL2 cells transfected with DRONC. Activation of DRONC was assessed by Western blotting with an antiserum that was generated against the carboxyl-terminal subunit of DRONC. This antibody was shown to pick up both transfected DRONC protein and purified DRONC antigen (data not shown) and recognises both full-length (p50) DRONC and carboxyl-terminal cleavage fragments. In control experiments, DRONC transfected SL2 cells were left untreated and expression of full-length precursor over a 24 h time period, is shown in
Figure 3.12 In vitro processing of Drosophila caspases.

The various recombinant Drosophila caspases were incubated with either $^{35}$S-DCP-1 (A), $^{35}$S-DCP-2 (B) or $^{35}$S-drICE (C) for 3 h at 37°C and processing analysed by protein electrophoresis and autoradiography. Note that DRONC was only able to process drICE but not DCP-1 or DCP-2.
Figure 3.13A. When cells were treated with apoptotic stimuli, DRONC processing was seen (Figure 3.13 B-C). Processing is evident by a decrease in DRONC 50kDa precursor and appearance of approximately 34 kDa and 24 kDa intermediates. The 36 kDa band corresponds to DRONC minus prodomain, which appears to be further processed at the amino-terminus to reveal a 24 kDa band. No smaller 14 kDa subunit could be detected, possibly due to rapid degradation of the protein. Interestingly, cycloheximide induced rapid processing and activation of DRONC, with the full length DRONC precursor almost undetectable 4 h after treatment (Figure 3.13C). In contrast, UV-irradiation did not induce complete processing of DRONC with the 36 kDa fragment appearing after 8 h and a decrease in precursor seen only at 16 h (Figure 3.13D). Thus, it appears that DRONC is processed during apoptosis by varying stimuli albeit with differing efficiency.
Figure 3.13 DRONC is processed in response to apoptotic stimuli.

SL2 cells were transfected with pRMHa3-DRONC, and after CuSO$_4$ induction, DRONC protein was analysed by Western blotting with α-DRONC antibody at 0, 2, 4, 8, 16 and 24 h without treatment with apoptotic stimulus (A) or after treatment with etoposide (40 μM) (B), cycloheximide (25 μg/μl) (C) or UV-irradiation (50 J/m$^2$) (D). In each case the 50 kDa DRONC precursor is processed to 36 kDa and 24 kDa bands. No smaller subunit bands could be detected.
3.10 Discussion

Caspases are classed on the basis of the presence of an amino-terminal prodomain. Class I caspases contain long prodomains, that may comprise protein-protein interaction motifs which enable caspase oligomerization or recruitment to specific death complexes where they are activated. Class II caspases have short or absent prodomains and appear to be processed and activated by upstream Class I caspases. Although there are nine mammalian caspases with a CARD domain, no Drosophila CARD-containing caspases had been previously described. In particular a CED-3/Caspase-9 homologue that, like Caspase-9, induces apoptosis by complexing with Apaf-1 via a CARD-CARD interaction, in a cytochrome c/dATP-dependent manner. This chapter has described the identification of DRONC, a novel Drosophila CARD-containing caspase. Based on the Drosophila genomic sequence database, DRONC appears to be the only CARD containing caspase in the fly and is therefore likely to be the true homologue of CED-3/Caspase-9. DRONC was subsequently identified and cloned by Meier and colleagues (2000), whose findings are consistent with data presented in the following chapters, and have contributed to the initial characterisation of DRONC function.

A novel and important finding is the induction of dronc expression by the steroid hormone ecdysone. Ecdysone mediates deletion of obsolete larval tissues such as midgut and salivary glands during developmental PCD. A large transient peak of ecdysone activity occurs before puparium formation and accompanies morphogenesis of the adult legs and wings as well as PCD of larval midgut (reviewed in Baehhcke 2000). Another pulse of ecdysone occurs during the prepupal-pupal transition, 10 h after puparium formation, and induces PCD of larval salivary glands, head eversion and adult differentiation. (reviewed in Baehcke 2000). Expression of dronc is up-regulated 12 h following puparium formation, coincident with the second peak in ecdysone activity. The demonstration that dronc mRNA can be dramatically upregulated by ecdysone suggested that it may be one of the primary mediators of PCD in larval midgut and salivary gland during metamorphosis. A recent publication by Lee and colleagues (2000) demonstrated transcriptional regulation of dronc by ecdysone-induced primary response genes, thereby providing a direct link between ecdysone activity and apoptosis of larval tissues. As
described in chapter 1, the ecdysone receptor complex, consisting of EcR and USP nuclear receptors, activates transcription of a number of early regulatory genes which in turn activate several 'late' genes that subsequently induce the morphogenetic changes associated with development of the adult fly (Jiang et al., 2000). Transcription of the E93 early gene is induced during the prepupal-pupal transition, in coordination with rpr and hid immediately before larval midgut and salivary gland cell death (Bahecke and Thummel, 1995). E93 is transcribed in a tissue- and stage-specific manner and a mutation in E93 results in ablation of salivary gland cell death and is larval lethal, suggesting it may be a critical regulator of ablation of steroid-mediated PCD of larval tissues (Lee et al., 2000). Interestingly, transcription of dronc is reduced in E93 mutant salivary glands 12 h following puparium formation, at the time when dronc mRNA is normally upregulated (Lee et al., 2000). This is the first indication that a caspase can be transcriptionally upregulated in response to a steroid hormone. The finding that dronc mRNA expression can be induced in midgut and salivary glands at earlier stages of larval development, indicates that all the components necessary for dronc transcription are present, but only activated in response to ecdysone during pupation. It appears that the concentration of E93 and subsequent transcription of dronc may determine the fate of salivary gland cells. The variable expression of dronc in other tissues, such as oocytes and eye discs, further suggests that DRONC may mediate tissue-specific cell death and possibly functions in other cell death pathways.

A second novel feature of DRONC is the active site amino acid sequence PFCRG surrounding the catalytic cysteine residue, which differs from the consensus QAC(R/Q/G)G. Each of the five Drosophila caspases described so far, differ from this consensus by only one residue but the QAC residues are completely conserved. Therefore the variation in the DRONC active site sequence may reflect unique substrate specificity. Our findings demonstrate that DRONC has a preference for pentapeptide sequences similar to VDVAD-amc, the optimal substrate specificity for Caspase-2. Using a combinatorial library, Hawkins and colleagues (2000) further defined the substrate specificity of DRONC. Interestingly, it was found that DRONC is not only able to cleave after aspartate residues, but has also acquired preference to cleave following glutamate
residues (Hawkins et al. 2000). Hawkins and colleagues (2000) demonstrated the ability of DRONC to self-process though cleavage after a glutamate residue (TQTE peptide sequence) where as its ability to process drICE is mediated though cleavage after the TETD sequence aspartate residue. Cleavage of DRONC at E352 correlates with the appearance of a 38 kDa fragment seen in our in vitro cleavage studies. This is the first finding that a caspase has novel cleavage site specificity distinct from the conserved aspartate residue. The specific physiological substrates for DRONC have not yet been determined but three common caspase substrates, drICE, lamin Dm0, and DREP-1 can all be cleaved by DRONC (this chapter, Hawkins et al. 2000, Meier et al., 2000), which indicates the ability of DRONC to cleave other caspases as well as various cellular substrates.

Consistent with its function as a caspase, our results demonstrate DRONC efficiently induces apoptosis in cell culture that is blocked by several caspase inhibitors. Interestingly, full length DRONC inefficiently induces apoptosis in SL2 cells but overexpression of an amino-terminal truncated form of DRONC(MPD) triggers apoptosis rapidly in these cells. Consistent with our findings, Meier and colleagues (2000) have shown that overexpression of DRONC(MPD) efficiently killed cultured cells. The finding that full length DRONC is unable to induce death in some cell lines (SL2 and Rat-1 cells), indicates that cleavage of the prodomain may be required for DRONC activation and apoptosis (this chapter and Meier et al., 2000). This may be reflective of the level of DRONC expression in some cell types, such that high levels of DRONC permits a localised protein concentration, thus enabling auto-proteolytic cleavage. Alternatively it is possible that the prodomain region can negatively regulate DRONC activity possibly though binding of an inhibitory molecule to DRONC-CARD. Such candidates for DRONC regulation are DIAP1 and DIAP2. In fact, Meier and colleagues (2000) have demonstrated the ability of DIAP1 to interact with the DRONC prodomain region. The finding that DIAP1 is still able to inhibit DRONC(MPD)-induced cell death may be explained by the weak interaction that is still detectable between DIAP1 and DRONC(MPD) (Meier et al., 2000) or by DIAP1 inhibition of the activity of downstream caspases such as drICE. Consistent with results from Meier and colleagues (2000), we
have shown that DRONC is able to process drICE \textit{in vitro}, so drICE acts as a putative downstream target of DRONC.

The processing of DRONC in response to various apoptotic stimuli demonstrated that it is activated early during apoptosis. This is consistent with the immediate activation of Class I caspases upon induction of cell death (Harvey \textit{et al.}, 1997). This activation may be mediated by interaction with adaptor proteins that couple these Class I caspases to signal transduction machinery, or to other molecules that function upstream in the apoptotic pathway. These interactions lead to the execution phase of apoptosis though activation of downstream Class II caspases (eg.drICE). In summary, results presented here describe the initial cloning and characterisation of DRONC activity. Further characterisation and biochemical analysis of DRONC regulation is presented in the following chapter.
Chapter 4

An essential role for the caspase DRONC in developmentally programmed cell death in *Drosophila*
4.1 Introduction

The identification of DRONC as a CARD-containing Class I caspase sparked much interest into its role in the *Drosophila* cell death pathway. In particular, the presence of a CARD-containing prodomain suggests that DRONC interacts with and is regulated by other *Drosophila* proteins, similar to the interaction between Caspase-9-CARD and Apaf-1, or Caspase-2-CARD mediated oligomerization. The most important starting point to characterise DRONC was to assess its interaction with all *Drosophila* mediators of cell death and determine its position in the death pathway. Results presented in this chapter also aimed to establish the mechanism of DRONC regulation by various inhibitors of cell death, and its effect on downstream executioners DCP-1 and dICE.

The genetic work described in this chapter was carried out in close collaboration with Dr Helena Richardson's laboratory at the University of Adelaide, Dept. of Genetics (now re-located to Peter MacCallum Institute, Melbourne). The procedure used in this study was to generate transgenic flies that ectopically express dronc in the *Drosophila* eye, and then cross these to flies that either ectopically express or contain deficiencies in the genes of various apoptotic regulators. The heterozygous progeny now contain half the dosage of each expressed gene and putative genetic interactions can be assessed by direct visualisation of the change in eye phenotype. The *Drosophila* eye provides a novel system that can be specifically targeted for expression of several genes under the control of the glass minimal region promoter (GMR). Furthermore, the fly eye is very sensitive to perturbation, so any phenotypic changes can be easily visualised, and the eye is not required for fly viability so effects can be seen in the developed adult fly.

Currently no *dronc* loss-of-function mutant exists, so we adopted the technique of RNA interference (RNAi) to ablate *dronc* function. The introduction of double stranded RNA (dsRNA) has been previously demonstrated to inhibit gene expression by 'post-transcriptional gene silencing' (Sharp 1999; Hammond et al., 2000). RNAi technique was first developed in *C. elegans* and has been used successfully in *Drosophila* and mammalian cells to specifically ablate gene function (Zhou et al. 1999, Colussi et al. 2000). Hammond and colleagues (2000) recently demonstrated that a 'loss-of-function phenotype' can be created in cultured *Drosophila* cells by introduction of specific dsRNA,
thereby providing a novel way to assess the effects of ablating the function of specific genes.

The aim of these studies was to delineate the biochemical function of DRONC and determine its position in the *Drosophila* apoptotic pathway by assessing its ability to interact with various death regulatory proteins. We have demonstrated that DRONC is an important mediator of cell death during *Drosophila* development and appears to be a key factor in cell death mediated by RPR, HID, GRIM and DARK. Most of the results described in this chapter have been published (Quinn et al. 2000).
Results

4.2 Ectopic expression of DRONC induces apoptosis in the Drosophila eye

To examine the in vivo effect of DRONC overexpression, transgenic flies were generated containing GFP-tagged wild type dronc or the inactive dronc mutant, dronc<sup>C118G</sup>, under the control of the yeast UAS(GAL4) promoter in pUAST vector. Expression of each construct can be targeted to different tissues by crossing flies to various GAL4 drivers. In this case, transgenic UAS-dronc and UAS-dronc<sup>C118G</sup> flies were crossed to flies containing the GMR-GAL4 driver to direct expression to the posterior region of third instar larval eye imaginal discs. DRONC protein expression was detected with α-GFP and α-DRONC antibodies (Figure 4.1A-D). High level of expression can be detected specifically in the posterior eye region, and this also demonstrates that the α-DRONC antibody generated in this study is able to pick up DRONC protein in situ. To determine whether ectopic expression of dronc could induce cell death, eye imaginal discs were stained with acridine orange to detect apoptotic cells. Wild type dronc expression induced massive cell death in the posterior region of the eye disc (Figure 4.1F) compared to dronc<sup>C118G</sup> expression (Figure 4.1E). To analyse the effect of dronc expression during embryogenesis, UAS-dronc and UAS-dronc<sup>C118G</sup> flies were crossed to the heat shock-inducible hsp70-GAL4 driver. As displayed in Figure 4.1G and H, dronc expression in stage 13 embryos shows an increase in ectopic cell death compared with dronc<sup>C118G</sup> expression, as visualised by TUNEL staining.

Expression of dronc in the adult eye is shown in Figure 4.2. The majority of GMR-GAL4;UAS-dronc flies died as early pupae, due to the inability of adults to break through the pupal case. The few flies that survived exhibited severely ablated eyes (Figure 4.2B) compared to dronc<sup>C118G</sup> expressing adults, which displayed normal eyes (Figure 4.2A). The phenotype generated by dronc expression is similar to that seen with rpr, hid and grim expression in the Drosophila eye (Grether et al., 1995; Chen et al.,1996; White et al.,1996).
Figure 4.1 *dronc* induces cell death in transgenic flies.

*UAS-dronc<sup>Cl8G</sup>-GFP* (A, C, E) or *UAS-dronc-GFP* (B, D, F) flies were crossed to *GMR-GAL4*, and the third instar larval imaginal eye discs were analysed.

(A-D) Third instar larval eye imaginal discs were co-stained with α-*DRONC* antisera (red) or α-GFP (dark blue) and stained for DNA using Hoechst 33258 (light blue).

(E and F) Eye imaginal discs stained with acridine orange (AO) to detect dying cells.

(G) *UAS-dronc<sup>Cl8G</sup>-GFP* or (H) *UAS-dronc-GFP* flies were crossed to flies containing the heat shock-inducible transgene *hsp70-GAL4*. Expression of *dronc* was induced in stage 13 embryos by heat shock for 30 min at 37°C, and apoptotic cells detected by TUNEL.
<table>
<thead>
<tr>
<th>$GMR$-dronc$^{C318G}$</th>
<th>$GMR$-dronc</th>
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</thead>
<tbody>
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<td><img src="image2.png" alt="Image B" /></td>
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<td><img src="image4.png" alt="Image D" /></td>
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<tr>
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<td><img src="image6.png" alt="Image F" /></td>
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<tr>
<td><img src="image7.png" alt="Image G" /></td>
<td><img src="image8.png" alt="Image H" /></td>
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</tbody>
</table>

- $\alpha$-DRONC
- $\alpha$-GFP
- AO
- embryo TUNEL
Figure 4.2 Ectopic expression of *dronc* induces death in the *Drosophila* eye.

(A) *UAS-dronc*\(^{C318G}-GFP\) or (B) *UAS-dronc-GFP* flies crossed to *GMR-GAL4* flies and adults analysed for their eye phenotype by scanning electron microscopy. Note the severely ablated eye phenotype, resulting from expression of *dronc*, from the few adult flies that survived.
GMR-dronc<sup>C318G</sup>  GMR-dronc
4.3 The caspase inhibitor P35 inhibits dronc induced death

Several transgenic lines of UAS-dronc flies resulted in less lethality when crossed to GMR-GAL4 and were used in studies to examine genetic interactions between DRONC and other apoptotic regulatory molecules. Two lines in particular (#23 and #80) were used for the generation of recombinants on the second chromosome with GMR-GAL4. When GMR-GAL4, UAS-dronc#80/+ flies were crossed to wild type (Canton-S) flies at 29°C, resulting adult flies displayed severely rough and mottled eyes due to ablation of pigment and photoreceptor cells (Figure 4.3A). Given that the baculovirus caspase inhibitor P35 is able to prevent DRONC-induced cell death in cultured cells, we assessed whether this was possible in vivo. Co-expression of p35, under the GMR promoter was able to suppress the GMR-dronc eye ablated phenotype at 29°C (Figure 4.3A). Therefore DRONC is sensitive to expression of P35 in the Drosophila eye.

To assess whether P35 could immunoprecipitate with DRONC, we transfected Drosophila SL2 cells with HA/His6-tagged DRONC and HA-tagged P35. As shown in Figure 4.3B, P35 was present in the DRONC immunoprecipitated complex indicating that these proteins can physically associate. Interestingly, P35 protein appeared to be processed in SL2 cells, which was previously shown to be essential for P35 binding and inhibition of caspases. We were not able to detect any processing of P35 by DRONC in vitro, and could not co-immunoprecipitate P35 and DRONC in 293T cells (data not shown), so it appears that another factor present in the SL2 lysate may be facilitating this interaction.

4.4 DRONC interacts with DIAP1 and DIAP2

The dronc eye phenotype was slightly improved when the temperature was decreased to 25°C (compare Figures 4.3A and 4.4A). It appears then that the dronc eye phenotype can be regulated in a temperature dependent manner, providing a means of regulating dronc expression levels, and therefore, a dosage-sensitive system for examining genetic interactions between dronc and other genes. To assess whether the GMR-dronc eye ablation phenotype was sensitive to halving the dosage of diap1, GMR-dronc flies were crossed to flies containing a deficiency of diap1 or a loss-of-function diap1 allele
Figure 4.3 *p35* suppresses the *GMR-dronc* eye ablation phenotype.

(A) *GMR-GAL4, UAS-dronc#80/Cyo* flies were crossed to flies ectopically expressing *p35* [+*p35*] at 29°C and the eye phenotype of the transheterozygous progeny was analysed by light microscopy. *(Left to right)* wild type (Canton-S) [wt], *GMR-GAL4, UAS-dronc#80/+ [dronc]* and *GMR-GAL4, UAS-dronc#80/+; GMR-p35/+ [+ *p35*].

(B) Lysates from SL2 cells co-transfected with HA- and -His6-tagged DRONC and HA-tagged P35 were precipitated with Talon resin and then western blotted (WB) with α-HA antibody *(upper panel)*. In a control experiment, lysates were immunoprecipitated (IP) and immunoblotted with α-HA *(lower panel).*
A

B

HA-DRONC-His<sub>6</sub>
P35-HA

TALON
WB: α-HA

IP: α-HA
WB: α-HA
Figure 4.4 dronc genetically and biochemically interacts with diap1 and diap2.

(A) GMR-dronc#80/CyO flies were crossed to flies containing either
- a deficiency in diap1: GMR-GAL4, UAS-dronc#80/+; Df(3L)brm11 [-diap1]
- a loss of function diap1 mutant: GMR-GAL4, UAS-dronc#80/+; thread5/+ [diap1<5]
- a deficiency in diap2: GMR-GAL4, UAS-dronc#80/Df(2R)Jp1 [-diap2]
- or to flies ectopically expressing diap1 or diap2:
  GMR-GAL4, UAS-dronc#80/+; GMR-diap1/+ [+diap1] and
  GMR-GAL4, UAS-dronc#80/+; GMR-diap2/+ [+diap2].

All crosses carried out at 25°C. Wild type (Canton-S) [wt] and GMR-GAL4, UAS-dronc#80/+ [dronc] fly eyes are shown in comparison.

293T cells were co-transfected with GFP-tagged DRONC and either MYC-tagged DIAP1 (B) or HA-tagged DIAP2 (C).

(B) Cell lysates were immunoprecipitated with α-MYC antibody (middle and bottom panels) and pelleted proteins were immunoblotted with α-GFP to detect DRONC (middle panel). (C) Cell lysates were immunoprecipitated with α-GFP antibody (top and middle panels) and protein complexes were immunoblotted with α-GFP antibody to detect DRONC (top panel), or with α-HA antibody to detect DIAP2 (middle panel).

In control experiments, DRONC, DIAP1 or DIAP2 protein expression was detected by immunoprecipitating and immunoblotting with α-GFP, α-MYC or α-HA antibodies respectively. The position of IgG heavy chain (IgG\textsubscript{H}), below DIAP1 and DIAP2 bands, is indicated.
Resulting heterozygote flies displayed a dominantly enhanced GMR-dronc eye phenotype at 25°C (Figure 4.4A). This cross also resulted in a 10-fold reduction in the number of GMR-dronc/+; Df(diap1)/+ adult flies generated, suggesting that a mutation of diap1 dominantly enhances lethality associated with GMR-dronc. In contrast, a deficiency removing diap2, did not have any significant effect on GMR-dronc phenotype (Figure 4.4A-lower panel). Thus diap1 but not diap2 interacts with dronc in a dosage-sensitive manner.

Interestingly, ectopic expression of GMR-diap1 or GMR-diap2 was able to suppress GMR-dronc eye phenotype (Figure 4.4A-lower panel). Although GMR-diap2 was less effective in suppressing this phenotype than GMR-diap1, it appears that both are able to directly or indirectly prevent DRONC-mediated cell death. A physical interaction between DRONC and DIAP1 or DIAP2 was assessed through immunoprecipitation assays. Co-transfection of 293T cells with DRONC-GFP and DIAP1-MYC or DIAP2-HA showed that both proteins were able to co-immunoprecipitate with DRONC (Figure 4.4B and C).

4.5 DRONC genetically interacts with the H99 genes

To assess whether the GMR-dronc eye phenotype was sensitive to the dosage of the H99 genes (reaper, hid and grim), GMR-dronc flies were crossed to a deficiency removing the H99 genes, Df(3L)H99, at 29°C (Figure 4.5A). The H99 deficiency dominantly suppressed the ablated phenotype of GMR-dronc (Figure 4.5A-top panel), suggesting that DRONC-mediated death is sensitive to the dosage of the H99 genes. A deficiency that removes a large part of chromosome 3 (region 67A2-67D13) containing the dronc gene, Df(3L)AC1, was used for further genetic interaction analyses. Halving the dosage of dronc significantly modified the ablated eye phenotype induced by ectopic expression of GMR-hid and GMR-rpr (Figure 4.5A-lower panel). Together, these findings demonstrated that DRONC acts as a downstream target of RPR, HID and GRIM.

Expression of transfected RPR, HID or GRIM in Drosophila SL2 cells was very low, possibly due to their ability to efficiently induce apoptosis in these cells together with the poor transfection efficiency of SL2 cells. Due to the inability to pick up expression of
Figure 4.5 dronc genetically interacts with the H99 genes and forms a protein complex with GRIM.

(A) GMR-dronc#80/CyO flies were crossed to flies containing a deficiency in the H99 genes: GMR-GAL4, UAS-dronc#80/+; Df(3L)H99+/+ [-H99], at 29°C (top panel).

GMR-hid/+ [hid] or GMR-rpr/+ [rpr] flies were crossed to flies containing a deficiency in the dronc gene: GMR-hid/+; Df(3L)ACI/+ [hid/dronc], GMR-rpr/+; Df(3L)ACI/+ [rpr/dronc] (bottom panel). Wild type (Canton-S) [wt] and GMR-GAL4, UAS-dronc#80/+ [dronc] fly eyes are shown in comparison.

(B) 293T cells were transfected with GFP-tagged DRONC alone or with either FLAG-tagged GRIM, RPR or HID. Cell lysates were immunoprecipitated with α-GFP antibody (top and middle panels) or α-FLAG antibody (bottom panel) and western blot analysis was carried out using α-FLAG antibody to detect GRIM, RPR and HID (middle and bottom panels). The position of IgG light chain (IgG\_\text{L}) is indicated. Immunoblot analysis with α-GFP shows DRONC protein expression (top panel). The middle panel does not show the upper part of the immunoblot as no bands corresponding to HID at 50 kDa were seen. The smaller band in GRIM transfected lanes is likely to be a proteolytic fragment of GRIM as noted by others (Claveria et al., 1998).
A

B

<table>
<thead>
<tr>
<th>Combination</th>
<th>DRONC-GFP</th>
<th>GRIM-FLAG</th>
<th>RPR-FLAG</th>
<th>HID-FLAG</th>
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</thead>
<tbody>
<tr>
<td>wt</td>
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<tr>
<td>rpr/dronc</td>
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**Immunoprecipitation (IP):**

- **α-GFP**
  - 78 kDa: DRONC
- **α-FLAG**
  - 16 kDa: GRIM
  - 50 kDa: IgG
  - 16 kDa: GRIM
  - 10 kDa: RPR
these proteins, 293T cells were used for further biochemical interaction studies. DRONC-GFP was co-transfected with either RPR-FLAG, GRIM-FLAG or HID-FLAG in 293T cells and as shown in Figure 4.5B, DRONC co-immunoprecipitated with GRIM, but not RPR or HID. Thus, the H99 protein GRIM may be the prime mediator of DRONC induced cell death by forming a complex with DRONC.

The localisation of ectopically expressed DRONC in 293T cells was also monitored when co-expressed with RPR, HID or GRIM. As previously noted, DRONC expression localises mainly to cytoplasm, with staining seen around the nucleus of some cells (Figure 4.6). Both RPR and GRIM localise mainly to cytosol whereas HID appears to be primarily localised to mitochondria, visualised as cytoplasmic aggregates concentrated around the nucleus (Figure 4.6, and Haining et al., 1999). DRONC was able to co-localise with GRIM, when overexpressed in 293T cells (Figure 4.7A) and co-localisation of DRONC with RPR in the cytosol was also detectable (Figure 4.7B). Interestingly, when co-expressed with HID, DRONC appears to be transported into mitochondria. Therefore, although HID has not been demonstrated to physically interact with DRONC, it is able to induce DRONC translocation to mitochondria in our overexpression experiments.

4.6 DIAP1 mediates an interaction between DRONC and GRIM

*In vitro* assays were carried out to establish wether DRONC could directly interact with the various apoptotic regulators, assessed above. DRONC-GST was unable to directly interact with the caspase inhibitor P35 (Figure 4.8). As a positive control, DIAP1 was shown to directly interact with GRIM. In contrast, GRIM could not directly interact with DRONC, but in the presence of DIAP1, a complex between GRIM, DIAP1 and DRONC could be seen (Figure 4.8- last lane). These findings suggest that DIAP1 mediates the interaction between DRONC and GRIM.
Figure 4.6 Cellular localisation of DRONC, GRIM, RPR and HID in 293T cells.

Expression vectors containing either GFP, DRONC-GFP, DRONC<sup>C318G</sup>-GFP or GRIM-FLAG, RPR-FLAG or HID-FLAG were transfected into 293T cells and 24 h post-transfection, cells were fixed and analysed for GFP expression [green] (top panel) or were fixed and stained with α-FLAG antibody followed by a mouse-rhodamine conjugated antibody [red] (lower panel) and examined by fluorescence microscopy. Cells were viewed under 100x magnification.
Figure 4.7 DRONC co-localises with HID in transfected 293T cells.

293T cells were co-transfected with DRONC-GFP and either GRIM-FLAG (A), RPR-FLAG (B) or HID-FLAG (C). 24 h post-transfection cells were fixed, permeabilised and stained with α-FLAG antibody, followed by a mouse-rhodamine conjugated antibody, and viewed by fluorescence microscopy. DRONC-GFP expression was visualised under a FITC filter (green) (left), GRIM-FLAG, RPR-FLAG and HID-FLAG expression is seen in red (middle) and merged images with overlapping green and red pixels appear yellow/orange (right).
Figure 4.8 The interaction between DRONC and P35 or GRIM is indirect.

Purified DRONC-GST was incubated with purified $^{35}$S-labelled P35-HA, GRIM-FLAG DIAP1-MYC. $^{35}$S-labelled proteins were visualised by autoradiography after immunoprecipitation with α-HA, α-FLAG or α-MYC (top panel). Binding of DRONC-GST was visualised by blotting with α-DRONC polyclonal antibody (lower panel). Small amounts of DRONC are observed binding to the sepharose beads alone (first lane). The lower molecular weight bands present in the DIAP1 lanes are likely to be breakdown products. The lower molecular weight bands seen with DRONC-GST correspond to cleavage products. DRONC does not co-immunoprecipitate with GRIM or P35, but comes down with GRIM in the presence of DIAP1 (last lane).
<table>
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<th>Construct</th>
<th>35S-P35-HA</th>
<th>35S-GRIM-FLAG</th>
<th>35S-DIAP1-MYC</th>
<th>DRONC-GST</th>
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<td>35S-P35-HA</td>
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<tr>
<td>78</td>
<td>35S-GRIM-FLAG</td>
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<tr>
<td>40</td>
<td>DRONC-GST</td>
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WB:α-DRONC

kDa
4.7 DRONC interacts with drICE

To analyse whether DRONC could interact with Drosophila Class II caspases, 293T cells were co-transfected with DRONC-GFP and drICE-HA and immunoprecipitated accordingly. drICE was able to co-immunoprecipitate with DRONC (Figure 4.9A). To determine whether this interaction was direct, in vitro translated 35S-drICE was incubated with purified DRONC-GST and proteins detected by autoradiography and α-DRONC immunoblotting respectively (Figure 4.9B). An interaction between DRONC and drICE was detectable in vitro (Figure 4.9B) suggesting drICE may act as a putative downstream target of DRONC. An interaction between DRONC and DCP-1 or DCP-2/DREDD was also analysed by immunoprecipitation assays, but a positive association could not be detected.

4.8 DRONC and DARK interactions

Since the cloning of DARK, it was of interest to determine whether DARK could form a complex with DRONC, thereby recapitulating the mammalian mitochondrial pathway and the interaction between Apaf-1 and Caspase-9. We first examined the genetic interaction between these two molecules by assessing whether a decrease in the dosage of dark modified the eye ablation phenotype of GMR-dronc at 29°C. Three different P-element allele mutants of dark, dark<sup>CD4</sup>, dark<sup>CD8</sup> and dark<sup>(2)k11502</sup> showed suppression of the GMR-dronc phenotype, the effect of the latter two are demonstrated in Figure 4.10A. These findings indicate that DARK promotes DRONC-induced cell death in the Drosophila eye.

The next objective was to determine whether DRONC and DARK could physically interact with each other through immunoprecipitation reactions. Due to the low expression of the large 170 kDa full length protein in both SL2 and 293T cells, we used a truncation mutant of DARK, comprising amino acids 1-411, and lacking the carboxyl-terminal WD40 repeats. The amino terminal region of DARK, containing only the CARD and CED-4/Apaf-1 homology domains, has been shown to bind to both DREDD and drICE (Rodriguez et al. 1999). To investigate whether truncated DARK could interact with DRONC, SL2 cells were co-transfected with a MYC-tagged DARK<sup>1-411</sup> and
Figure 4.9 DRONC interacts with drICE.

(A) 293T cell lysates transfected with DRONC-GFP alone or with drICE-HA, were immunoprecipitated with α-GFP (top panels) or α-HA (bottom panel), and immunoblotted with α-GFP to detect DRONC expression (top panel) or with α-HA to detect drICE (lower panels). drICE was detected as a complex with DRONC-GFP (middle panel). The position of IgG heavy (IgG_H) and IgG light (IgG_L) bands are indicated.

(B) A direct interaction between DRONC and drICE was assessed in vitro by incubating purified DRONC-GST with ^35S-labelled drICE-HA. ^35S-labelled drICE-HA was immunoprecipitated with α-HA antibody and visualised by autoradiography (top panel). Binding of DRONC-GST was visualised by immunoblotting with α-DRONC polyclonal antibody. Residual DRONC-GST is seen binding to sepharose alone (first lane).
A

DRONC-GFP

kDa

IP: αGFP

WB: αGFP

WB: αHA

IP: αHA

WB: αHA

B

35S-drlCE-HA

DRONC-GST

kDa

IP: α-HA

WB: α-DRONC

WB: α-DRONC-GST
Figure 4.10 DRONC interacts with DARK.

(A) GMR-dronc#80/CyO flies were crossed with dark P-element mutants [-dark(2)kl1502 or -darkCD8] at 29°C, and the eye phenotype of the transheterozygous progeny analysed.

-(top panel): wild type (Canton-S) [wt] and GMR-GAL4, UAS-dronc#80/+ [dronc]
-(bottom panel): GMR-GAL4, UAS-dronc#80/+; dark(2)kl1502/+ [dark(2)kl1502] and GMR-GAL4, UAS-dronc#80/+; darkCD8/+ [darkCD8]

(B) SL2 cell lysates transfected with HA-tagged DRONC alone or with MYC-tagged DARK1-411, were immunoprecipitated with α-MYC antibody (middle and bottom panels). DRONC protein was detected in lysate (top panel) and in the DARK immunoprecipitated complex (middle panel) by western blotting with α-HA antibody. DARK1-411 protein expression was detected by immunoprecipitating and immunoblotting with α-MYC antibody (bottom panel)
HA-tagged DRONC construct. DARK-MYC was immunoprecipitated with αMYC antibody and complexes analysed by immunoblotting with α-HA antibody (Figure 4.10B). DRONC-HA protein was clearly detected in the DARK immunoprecipitate, establishing that the amino-terminal region of DARK, containing only CARD and CED-4/Apaf-1 homology domains, is sufficient for association with DRONC.

4.9 DARK is important for DRONC processing

To test the requirement of DARK for DRONC activation, extracts from dark^{CD8} homozygous flies were prepared and assessed for their caspase activity and ability to cleave DRONC in vitro. As shown in Figure 4.11A, dark mutant flies had reduced caspase activity compared with wild type on DEVD-amc, VDVAD-amc and VEID-amc peptide substrates. The previous chapter demonstrates the preferred substrate for DRONC as VDVAD-amc, and during the course of this study Hawkins and colleagues (2000) defined the preferred substrate specificity of DRONC as VEID-amc, a Caspase-6 substrate cleavage sequence found in nuclear lamins. It was found that dark mutant flies display markedly low VEID activity, which may imply reduced DRONC activity in these flies. DEVD is a Caspase-3 substrate that is cleaved poorly by DRONC but preferred by the downstream caspases DCP-1 and drICE, so the lack of activity on this substrate implies absence of activation of Class II caspases in dark mutant flies. Thus, dark mutant extracts contain lower cleavage activity toward both preferred DRONC substrates and preferred downstream caspase substrates. Kanuka and colleagues (1999b) also observed lower caspase activity in extracts prepared from dark mutant embryos, so it is clear that DARK mediated death requires the activation of caspases. Furthermore, we demonstrated that dark mutant extracts show considerably reduced ability to cleave DRONC to its active form (Figure 4.11B), suggesting that DARK is important for DRONC processing. dark^{CD8} is a hypomorph mutant and it is unclear whether this is a null mutant because a deficiency of the dark region is not available, so the residual DRONC processing observed may be due to residual DARK activity or to an alternative mechanism.
Figure 4.11 DARK is required for DRONC activation.

(A) $\text{dark}^{CD8}$ has lower caspase activity. Caspase activity from $\text{dark}^{CD8}$ mutant flies was compared with wild type ($\text{w}^{118}$) flies by incubation with 100µM various fluorogenic caspase substrates, VDVAD-amc, DEVD-amc or VEID-amc at $37^\circ$C for 30 min. The release of -amc was monitored on a fluorimeter. An equivalent amount of protein was used in each sample. $\text{dark}^{CD8}$ flies consistently had lower activity on the substrates assessed. Bars represent average fluorescence units ± SEM derived from three independent experiments.

(B) $\text{dark}^{CD8}$ mutant extract has reduced ability to process DRONC. Protein lysates from $\text{w}^{118}$ or $\text{dark}^{CD8}$ flies were incubated with in vitro translated $^{35}$S-labelled DRONC for 3 h at $37^\circ$C and cleavage products were assessed by autoradiography. An equivalent amount of protein was used in each reaction. $\text{dark}^{CD8}$ mutant fly extracts were less efficient in processing DRONC. Control experiments contained DRONC with buffer only. Full length DRONC is indicated with an arrow, asterisks indicate DRONC cleavage products.
**A**

![Bar Graph](image)

- VDVAD-amc
- DEVD-amc
- VEID-amc

**B**

![Western Blot](image)

**kDa**

- 50
- 32
- 22
- 18
- 14

**35S-DRONC**
4.10 Cytochrome c enhances processing and activation of DRONC

The activation of caspases by DARK has been found to be dependent on the binding of cytochrome c to WD40 repeats, similar to the manner of cytochrome c activation of Apaf-1 (Kanuka et al. 1999b). Due to technical difficulties associated with producing active DARK, and the low levels of expression of full-length DARK in transfected cells, we were unable to assess any direct activation of DRONC by DARK or look at direct complex formation.

To assess whether cytochrome c was necessary for the processing of DRONC, in vitro translated, 35S-DRONC-HA was purified by immunoprecipitating with α-HA antibody, and then incubated with SL2 cytoplasmic extract in the presence or absence of cytochrome c/dATP (Figure 4.12). The addition of cytochrome c and dATP to the cleavage reaction was able to enhance the processing of DRONC, suggesting that cytochrome c may play a role in DRONC activation during apoptosis.

4.11 DRONC is essential for embryonic cell death.

Specific mutations in DRONC are currently unavailable, so we used the technique of RNAi to ablate dronc gene function during embryogenesis. dronc double stranded mRNA was injected into pre-cellularised embryos and samples were aged until stage 13. Embryos were analysed by α-DRONC antibody staining to assess the efficiency of DRONC protein ablation and TUNEL assays revealed the number of apoptotic cells. At stage 13, uninjected embryos show DRONC expression throughout the embryo, and contain a large number of TUNEL-positive cells (Figure 4.13A and C). In contrast, in stage 13 dronc RNAi injected embryos, DRONC protein was undetectable and very few TUNEL-positive cells were present (Figure 4.13 B and D). At least 400 dronc RNAi-injected embryos were analysed and results were consistent for all embryos. Although dronc RNAi-injected embryos failed to hatch, examination of embryonic structures by Nomarski optics showed no apparent gross structural defects.

Embryos were also stained with a neural differentiation marker monoclonal antibody 22C10, to reveal whether ablation of dronc was affecting neural development. As shown in Figure 4.15E and F, neural differentiation overall, appeared normal. Higher
magnification view of the thoracic region indicated normal development of neuronal differentiation (data not shown). Together these results suggest that dronc is essential for cell death during embryogenesis. DRONC shares limited sequence homology with other Drosophila caspases, so it is unlikely that dronc RNAi effects the function of other caspases, thus all affects seen here are due to the ablation of dronc function alone.
4.12 Cytochrome c enhances processing of DRONC.

*In vitro* translated $^{35}$S-labelled DRONC-HA was purified by immunoprecipitation with $\alpha$-HA antibody. Purified $^{35}$S-DRONC was incubated with buffer or an SL2 cell extract in the absence (-) or presence (+) of cytochrome c (1µM) and dATP (2mM) for 2 h at 37°C. Processing of $^{35}$S-DRONC was visualised by autoradiography. Processing of DRONC is slightly enhanced in the presence of an SL2 extract with the addition of cytochrome c / dATP.
lysate
cytochrome c / dATP
kDa
14
50
36
<
3sS-DRONC
Buffer SL2
+ + ± ±
* * * *
3sS-DRONC
Figure 4.13 DRONC is required for cell death in embryos.

RNAi was used to ablate dronc function in embryos. Uninjected embryos (A, C, E) or pre-cellularized embryos injected with double stranded dronc RNA (B, D, F) were aged to stage 13 before fixation and staining for TUNEL (A, B), α-DRONC antibody (C, D) or the neural differentiation marker Mab 22C10 (E, F). (G, H) Higher magnification views of the thoracic region of an uninjected embryo and a dronc double-stranded RNA injected embryo respectively, stained with TUNEL. (I) Buffer injected control of a stage 13 embryo.
<table>
<thead>
<tr>
<th>uninjected</th>
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<td>C</td>
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- **TUNEL**
- $\alpha$-DRONC
- Mab 22C10
4.12 Discussion

This chapter demonstrates that DRONC mediates PCD \textit{in vivo}. In summary, DRONC overexpression results in ectopic cell death in transgenic flies in different tissues and at various developmental stages. Conversely, ablation of DRONC in early embryos using RNAi results in dramatic decrease in the number of apoptotic cells during embryogenesis. DRONC is therefore an important mediator of PCD during \textit{Drosophila} development. Although ectopic overexpression of DRONC resulted in substantial cell death, not all cells expressing DRONC underwent PCD. This indicates that overexpression of DRONC alone is insufficient to kill cells and may require other factors to activate DRONC itself so that it can in turn activate downstream caspases. The RNAi studies described were only able to demonstrate the importance of DRONC during embryonic PCD as none of the injected embryos developed into larvae. It would therefore be of interest to see whether DRONC is essential for PCD in tissues during larval and pupal development and in the adult fly.

The interaction between DRONC and other components of the apoptotic machinery have been analysed through genetic and biochemical means. This study has demonstrated that the \textit{GMR-dronc} eye phenotype can be suppressed by \textit{GMR-p35} and is sensitive to the dosage of the caspase inhibitor \textit{diap1}, by the \textit{H99} genes and by \textit{dark}. The inhibition by P35 is consistent with results from chapter 3, where P35 was able to inhibit both full length DRONC and a truncated version of DRONC, lacking the pro-domain, when co-expressed in cultured cells. Furthermore, DRONC can form a complex with P35, GRIM, DARK, DIAP1 and DIAP2 but not with HID or RPR. We also showed that extracts from \textit{dark} mutant flies have reduced caspase activity and decreased ability to cleave DRONC and, that cytochrome \textit{c} enhances DRONC processing to some extent, suggesting that DARK and cytochrome \textit{c} are important for DRONC activation.

The suppression of \textit{GMR-dronc} phenotype by co-expression of the caspase inhibitor P35 and the biochemical interaction observed between DRONC and P35 demonstrate that DRONC-induced cell death is caspase-dependent. This finding was confirmed by Jones and colleagues (2000), who found inhibition of DRONC-mediated death in S2 cells by P35. At the time of completion of these experiments, two additional
publications demonstrated that DRONC is an apical P35-insensitive caspase, and that genetic studies did not display interactions between these two molecules (Hawkins et al. 2000; Meier et al. 2000). The differences in genetic interactions may simply be due to the level of expression of such genes; our GMR-dronc eye ablation phenotype being less severe than the phenotype assessed in these latter studies, and therefore more likely to reveal subtle genetic interactions. The difference between our biochemical data may be explained by differences in experimental design, as the studies by Meier et al. (2000) and Hawkins et al. (2000) were carried out in yeast in which no other apoptosis components are present. Our studies do not demonstrate a direct interaction between DRONC and P35 in vitro, so it is likely that P35 is interacting with and inhibiting a downstream caspase rather than DRONC itself. A putative candidate is drICE, which can interact with, and is inhibited by P35 (Hawkins et al. 1999). Results presented here indicate that drICE can also interact with DRONC, consistent with findings by Meier and colleagues (2000).

Based on homology and the ability of DRONC to complex with DARK, DRONC is a likely functional homologue of CED-3/Caspase-9 and acts downstream of DARK and the H99 gene products. In support of this hypothesis, and consistent with other findings, we have shown that a deficiency that removes dronc, or expression of the dominant negative dronc mutant, is able to suppress the eye ablation phenotype of GMR-hid and GMR-rpr (this chapter, Hawkins et al. 2000; Meier et al. 2000). Because overexpression of apical caspases generally results in autoactivation, the ectopic expression of dronc was expected to be epistatic to the H99 genes and dark. However, our results demonstrate, that halving the dosage of the H99 genes and dark are rate-limiting for dronc function. An explanation for this may be that DRONC is unable to autoactivate very efficiently, even when overexpressed, and so may be dependent on the dosage of upstream activating genes.

Consistent with the genetic interaction, DRONC forms a complex with GRIM when co-expressed in cultured cells. However, this interaction is indirect and appears to occur through DIAPI, which can directly bind to both DRONC and GRIM. The significance of the in vivo interaction between DRONC and GRIM is unclear and requires further investigation. Another obscure finding is the co-localisation of DRONC with HID.
to mitochondria. Because DRONC and HID cannot interact in vivo, it is possible that another molecule acts as a chaperone between these two proteins. The reason for transport to mitochondria is unknown. Co-expression of Bcl-xL can inhibit mitochondrial localisation of HID, and prevent HID-mediated apoptosis (Hsu et al., 1997). However, it is unclear whether mitochondrial localisation of DRONC is required for its apoptosis-inducing activity.

Given that DRONC is a putative CED-3/Caspase-9 homologue, activation of DRONC is expected to require DARK function. Consistent with this notion, DARK can form a complex with DRONC in SL2 cells. Kanuka and colleagues (1999b) supported these findings by demonstrating that DRONC and DARK can interact in mammalian 293T cells and that this results in generation of the cleaved, active form of DRONC. In addition, we have shown that dark mutant extracts contain reduced levels of active caspases, and are impaired in their ability to generate the cleaved active form of DRONC. DRONC processing is influenced by cytochrome c activity, possibly through DARK, further suggesting that DRONC is a likely functional homologue of CED-3/Caspase-9 because it is can activated by DARK and cytochrome c. In addition the amino-terminal region of DARK is sufficient for binding to DRONC, indicating that binding may be mediated through CARD-CARD interactions, similar to Apaf-1 and Caspase-9.

The Drosophila inhibitor of apoptosis DIAP1 inhibits the activity of drICE and DCP-1 and is antagonised by RPR, HID and GRIM (Hawkins et al. 1999; Wang et al. 1999). Our data show a dosage-dependent enhancement of GMR-dronc by a diapl mutation and additionally show that DRONC and DIAP1 form a complex in vivo, consistent with a function of DIAP1 as an inhibitor of DRONC. Meier and colleagues (2000), also demonstrated a genetic and biochemical interaction between DRONC and DIAP1 and, furthermore, that the prodomain region of DRONC is required for the binding of DIAP1. Interestingly, results in chapter 3 demonstrate the ability of DIAP1 to efficiently inhibit death mediated by DRONC(MPD) in SL2 cells, so it is possible that DIAP1 can also bind to activated DRONC as a point of regulation in the pathway, or may simply ablate DRONC-induced death by inhibiting downstream caspases. We also observed binding between DIAP2 and DRONC. In addition, expression of diapl or diap2
was able to suppress the GMR-dronc phenotype, indicating that DIAP2 as well as DIAP1 can prevent DRONC-mediated cell killing. However, because a diap2 deficiency did not show a dominant enhancement of GMR-dronc, and DIAP2 is ineffective in preventing DRONC-induced cell death in cultured cells (chapter 3), it is likely that the suppression of GMR-dronc by GMR-diap2 is indirect, perhaps by inhibition of downstream caspases. The genetic data and biochemical observations presented in this chapter, highlighting a role for DIAP1 but not DIAP2 in suppressing DRONC function, are consistent with previous findings showing that diapl and diap2 function differently in inhibiting death (Hay et al. 1995; Wang et al. 1999). Halving the dosage of diap1, but not diap2, enhances rpr-, hid- or grim-induced cell death, whereas overexpression of diap1 or diap2 can inhibit rpr- or hid-induced cell death in the Drosophila eye (Hay et al. 1995; Wang et al. 1999). However, only overexpression of diap1 is able to prevent grim-induced cell death (Wing et al. 1998). The precise roles of DIAP1 and DIAP2 in the Drosophila apoptotic pathway are still unclear.

In summary, the results presented in this chapter show that DRONC is essential for cell death in early embryos and that ectopic expression of DRONC can induce cell death in flies. This data also provides evidence that DRONC is a functional homologue of CED-3/Caspase-9 in flies. The position of DRONC in the fly apoptotic pathway is depicted in Figure 4.14. DRONC, as a CARD-containing Caspase-9 homologue, is expected to function downstream of death signals and upstream of effector caspases such as drICE and DCP-1. The activation of DARK is proposed to lead to the activation of DRONC, which can be inhibited by DIAP1. The genetic and biochemical interactions between DRONC and P35, GRIM, DIAP1, DIAP2, drICE and DARK provide a solid framework for further investigation of the PCD pathway in Drosophila.
Figure 4.14 Position of DRONC in the *Drosophila* apoptotic pathway.

Proteins that have been shown to genetically interact with DRONC are highlighted in green, proteins that we have been shown to physically interact with DRONC are highlighted in blue. Death signals induced by RPR, HID and GRIM lead to the activation of caspases. DRONC, a CARD-containing CED-3/Caspase-9 homologue, is activated by the CED-4/Apaf-1 homologue DARK, which is required for RPR, HID, or GRIM induced cell death. DIAP1 acts by binding to procaspases and prevents their activation. RPR, HID and GRIM can also bind to DIAP1 and disrupt DIAP1-caspase complexes, leading to caspase activation. The baculovirus protein P35 acts to inhibit many caspases but has not been shown to directly inhibit DRONC. In the DRONC pathway, P35 may function by inhibiting a downstream caspase such as drICE.
Death signals

\[ \rightarrow \text{RPR} \rightarrow \text{HID} \rightarrow \text{GRIM} \rightarrow \text{DARK} \rightarrow \text{DRONC} \rightarrow \text{Effector caspases (drICE)} \rightarrow \text{Apoptosis} \]

DIAP1

? \rightarrow \text{Debcl}

P35
Chapter 5

Identification of DECAY, a novel Drosophila caspase related to mammalian caspase-3 and caspase-7
5.1 Introduction

The results presented in the preceding chapters have focussed on the initiator Class I caspase DRONC. Prior to this study, two Class II effector caspases, DCP-1 and drICE, had been described (Fraser and Evan, 1997; Song et al., 1997). As discussed in chapter 4, at least drICE is a direct target of DRONC-mediated activation in the caspase cascade. Both DCP-1 and drICE are poor inducers of apoptosis when overexpressed in cultured cells, but drICE significantly enhances the level of death mediated by expression of RPR, or treatment with cycloheximide or etoposide (Fraser and Evan 1997). Processing of drICE has been demonstrated during apoptosis, and although this has not been shown for DCP-1, both these caspases are able to process and activate each other in vitro. Furthermore, the processing of PARP, lamin Dm0 and ICAD by DCP-1 and drICE, gives support to the notion that they function as executioner Class II caspases during apoptosis.

To fully understand the role of various caspases in cell physiology, it is important to identify all caspases in a given model organism. This describes the identification and initial characterisation of a Class II caspase, termed DECAY. Results from this chapter have been published (Dorstyn et al., 1999).
Results

5.2 Identification of DECAY

We set out to search for new molecules with homology to various mammalian caspases. Using a TBLASTN program, we identified an expressed sequence tag in GenBank database, which encoded a partial caspase-like protein. Sequencing of the entire clone revealed a cDNA open reading frame of 287 amino acid residues with high degree of homology to the Caspase-3 subfamily. This molecule was named DECAY for Death Executioner Caspase related to Apopain/Yama. The full length decay DNA sequence has been deposited in GenBank (accession number AF 130469). Alignment of the DECAY sequence to the Drosophila genome indicated the presence of 80 extra base pairs at the 5' end of the gene. Amplification and sequencing of decay from a Drosophila embryonic cDNA library detected a stop codon further upstream, indicating the true ATG start was indeed further 5' of the coding sequence present in the EST. The genomic sequence of decay comprises a 941 base pair coding sequence that is split into three exons (Figure 5.1A). The complete mRNA coding sequence is displayed in Figure 5.1B. The chromosomal location of DECAY was determined by hybridisation to a filter comprising Drosophila genomic P1 clones. The decay gene was localised to the Fasl contig on chromosome 3, region 89C6-D4 (data not shown). None of the other published Drosophila caspase genes map to or near this region.

DECAY encodes a 308 amino acid protein (Figure 5.2A-C). In vitro translation of DECAY produced a 35 kDa protein consistent with its expected size (Figure 5.2B). DECAY shares greatest homology, 39% identity (54% similarity), with Spodoptera frugiperda Caspase-1. Of the mammalian caspases, DECAY shares approximately 37% identity (56% similarity) with Caspases-3 and -7. An alignment of all the Drosophila caspases showed that DECAY shared highest homology with DCP-1 and drICE, sharing 35% identity and 53-55% similarity (Figure 5.2D and 5.3). Similar to DCP-1 and drICE, DECAY lacks a long amino-terminal prodomain suggesting that it is a Class II downstream effector caspase. Interestingly, DECAY shares only 26 % identity with the remaining Drosophila Class II caspase DAMM and is only very distantly related to
Figure 5.1 Genomic structure and sequence of *decay*.

(A) The *decay* gene is located on chromosome 3, region 89C6-D4. Non-coding regions are shown as open boxes. *decay* gene is encoded by three exons, shown in blue.

(B) Sequence of *decay* cDNA. Coding region is highlighted in bold. Intron insertion sites are indicated with red arrows.
A

5' UTR  exon 1  exon 2  exon 3  3' UTR

1  331  909  1241  1547  1809

500 bp

B

1  ggacgtcag  aaatgacga  caccgacttc  tcgctttctg  ggacagaa  aa caccaag
61  aaggcaag  cggatgcac  caagatgcc  ctaagcccc  cattgcagct  ggatctcaa
121  aggatcataa  tctcgcggcc  caaacaag  cagacatag  agaattgcgc  ggcagcgccc
181  attcgcctaa  tctcgaacca  caagatgac  aagcagalaga  agtggagcg  ggcaccc
241  cggtatcgcc  atgacatgga  ggcacgtcgt  gatggatctg  atctgtatgt  aagcacttc
301  gacgatctga  cctctcctga  gatcagcgcac  agcgtcaaaag  aggctgctcg  ggaggatcc
361  agatcacaac  atgctttgct  gttgcagctg  atgctcagcc  gtcacgagag  caaagtcac
421  gctaaagca  gttctatgcc  tgtggaggcgc  cttttgaaatac  cttctctcgg  gacaacctgc
481  aagcgcctca  aagacaaacc  caaactctcttc  ttcctcaggg  cggcacccgtg  aagctcctcg
541  gaaaaagccag  tcggatttcct  cagctctctg  gtgcggactaca  gggagactgt  cccggagccc
601  gctgcgacggctcagcattcc  cacctatgac  atcctcatc  cggacgcagc  tgggtgtcttc
661  tattccatactc  tcacagcttc  gtttcaccttc  gtaaagcttg  agaacgtgatc  gctgagttcatc
721  cagagcctgt  tccgaggctcc  gacccagaggcc  gggggcaggc  aagctgcgatc  ggcacgctg
781  gatcggctatactctcattc  gacgagctgtg  ataaggaagg  tgggcctacagca  gtacagctcgc
841  aatagcgaaga  acgagcctgct  caacacagtag  aaggaatgc  ccaactttat  gttggactgg
901  aacaacaactc  tcagagtctgct  gttcgcccc  gagggagtga  cttcgcgtc  aacatcgagc
961  acaatcctattggtg  agttcagccgtg  cgggtcaaga  aagcagcagc  ccaacacctct
1021  ttcattttcatg  gtaatttggtt  gcaatgttttc  aatgaaacata  catatcgtg  gccgtttaaa
1081  aaaaaaaaaa  aaaaaa
Figure 5.2 DECAY sequence and its homology to other Drosophila caspases.

(A) Deduced amino acid sequence of DECAY consists of 308 amino acid residues. The pentapeptide sequence QACRG is shown in bold with the catalytic cysteine (aa150) residue highlighted red. (B) In vitro translation of DECAY shows that protein is 35 kDa in size. (C) Schematic representation of DECAY protein structure. Putative caspase cleavage sites are indicated that may generate large and small subunits. The catalytic active site is show as a grey box. (D) Phylogenetic relationship between all Drosophila caspases.
A

MDTDFSLFG QKNHKKDKA DATKIAHTPT SELDLKRII SRPTNETDYE NCARAGIALI 60
LNHKDVKGK QRGTERDRD DMRATLQCGF FDVTFDPLT FSEINDTLKE VAREDSQND 120
CFVLAVMSHG TEGKYYAKDM SYPVERLWNP FLGDNCNTLK NKPKLFFIQ ACRGANLEKAV 180
EFSSFAVMTR ELVPEPAAV QPITYAIPST ADILVFYSTF DKKFSSFRNVN DSWSFIIQSLC 240
RVLDQAAANE AATPE庖VELL RLLTAVNRKV AYEYQSNTKN EALNQMKEFMPNFMSTLTKTF 300
QLRVQPKPT

B

35 kDa

35S-DECAY

C

D

DAMM

STRICA

DRONC

DCP-1
drlCE

DECAY

DCP-2 / DREDD
Figure 5.3 Alignment of *Drosophila* caspases.

An amino acid sequence alignment of the seven *Drosophila* caspases using CLUSTAL W program at the European Bioinformatics Institute. Residues conserved in 6-7 caspases are highlighted in blue. Residues conserved in at least 5 caspases are highlighted in red and similar residues are shaded grey.
Drosophila Class I caspases, sharing only 27% identity and 43-45% similarity to DREDD, STRICA and DRONC (Figure 5.2D). DECAY is the only Drosophila caspase containing the conserved active site QACRG sequence encompassing the catalytic cysteine residue, which is found in most mammalian caspases.

5.3 decay mRNA expression during Drosophila development

RNA blot analysis detected a decay transcript of approximately 1.1 kilobase in size in most developmental stages; larvae, pupae and adult fly (Figure 5.4A). Highest expression of decay was detected during early third instar larval stage when developmental apoptosis is occurring. Due to low levels of decay during embryogenesis, we used poly A+ RNA in Northern blots. As demonstrated in Figure 5.4A (second panel), expression during embryogenesis is very low, but relatively high levels of decay transcript were detected in the adult fly. A direct comparison of decay expression levels throughout all developmental stages can be seen in RT-PCR analysis (Figure 5.4C). The presence of decay in early embryos suggests that it is maternally deposited into the embryo, because zygotic expression does not begin until stage 5.

We further analysed the expression pattern of decay during fly development by in situ hybridisation to Drosophila embryos and larval tissues using a digoxigenin-labelled antisense mRNA probe (Figure 5.5). Consistent with Northern blot data, decay is expressed at low levels throughout embryogenesis and shows no specific upregulation at stage 11, when PCD is first detectable in Drosophila (Figure 5.5A-C). In stage 6-7 cellularized embryos, decay mRNA is ubiquitously expressed (Figure 5.5B), but later stages show high level of decay expression in the gut (Figure 5.5C). We also examined the level of decay expression in third instar larval tissues and during oogenesis (Figure 5.5 E-L). Interestingly, a high level of expression was observed in the salivary glands and midgut tissue from third instar larvae (Figure 5.5E and F), preceding the onset of apoptosis of these tissues which occurs after pupariation (Jiang et al., 1997). The high expression levels of decay in third instar salivary gland and midgut mimics dronc expression in these tissues (chapter 4) and follows the peaks of ecdysone at this stage. We therefore set out to
Figure 5.4 Expression of decay mRNA.

(A) Northern blot analysis of decay expression using total RNA (left) or poly A\(^+\) enriched RNA (right) decay transcript is detected as a single band of approximately 1.1 kb in size. The lower panels depict ethidium bromide-stained gels corresponding to ribosomal RNA bands prior to membrane transfer. (B) RT-PCR analysis of decay expression. PCR was carried out on cDNA from various *Drosophila* developmental stages, using primers that generated a 0.95kb decay fragment. Lower panel shows a rp49 control RT-PCR on each sample.
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- 0.95 kb
- 0.45 kb

- decay
- rp49
Figure 5.5 *In situ* mRNA analysis of *decay* expression during *Drosophila* development.

*decay* mRNA was detected by *in situ* hybridisation with a digoxigenin-labelled anti-sense mRNA probe.

(A) Stage 5 syncitial embryo showing uniformly low levels of *decay* expression.

(B) Stage 7 embryo showing *decay* expression throughout the embryo. Regions of higher staining are due to tissue folding.

(C) Stage 13 embryo showing higher *decay* staining in the middle section, corresponding to gut tissue.

(D) Stage 8 embryo hybridised with a *decay* sense control probe.

(E) Third instar larval salivary gland showing high levels of *decay* mRNA.

(F) Third instar larval midgut showing high levels of *decay* expression.

(G) Late third instar larval eye imaginal disc with very low levels of *decay* expression.

(H) Brain lobes from third instar larvae with ubiquitous low levels of *decay* mRNA.

*decay* sense control on third instar larval tissues showed no staining (data not shown).

(I) Stage 10a adult egg chamber showing high expression of *decay* in nurse cells (*left*) but not the oocyte (*right*).

(J) Adult egg chambers showing an increase in *decay* mRNA expression at stage 9 compared to earlier stages.

(K) Hoechst 33258 staining of DNA in adult egg chambers showing nuclear morphology. At stage 12 the nurse cells are seen undergoing apoptosis (large nuclei on left of stage 12 egg chamber), and *decay* mRNA has been dumped into the oocyte (*J*). The oocyte is surrounded by follicle cells, whereas the germinal vesicle is out of the plane focus.

(L) *decay* sense control probe on adult egg chambers.
determine whether decay expression is also induced by ecdysone. However, no induction of decay mRNA was seen in ecdysone treated salivary gland or midgut (data not shown) compared to the massive up-regulation of dronc under the same conditions (chapter 4).

Only very low levels of decay expression were observed in third instar larval eye imaginal discs and brain lobes (Figure 5.5G and H) which contain apoptotic cells at this stage. However, upregulation of decay was not seen in eye disc or brain lobe cells undergoing apoptosis. During oogenesis, decay mRNA is detected in egg chambers of all stages and was present in nurse cells at high levels after stage 10a (Figure 5.5I). In stage 12 egg chambers, decay expression was absent from nurse cells undergoing apoptosis and present in the developing oocyte (Figure 5.5J and K). This finding is consistent with the dumping of the nurse cell cytoplasm into the oocyte during oogenesis, and the apoptosis of nurse cells that occurs at this stage.

5.4 Ectopic expression of DECAY in cultured cells

Many caspases, when overexpressed in cultured cells, induce apoptosis to some degree. We therefore analysed whether DECAY was able to induce apoptosis in transfected cells. In 293T cells, at either 24 or 48 h following transfection, full length DECAY was unable to induce a significant level of apoptosis. In contrast, expression of DECAY(MPD) induced approximately 35% death after 24 h, compared to vector transfected cells (Figure 5.6A). Cells transfected with the catalytic cysteine mutant (DECAY\(^{\text{C150G}}\)) did not induce death, indicating that the induction of cell death by DECAY is mediated though its cysteine protease activity (Figure 5.6A). In NIH-3T3 cells, only a small number of apoptotic cells were detected 24 h after DECAY transfection and this did not increase at 48 h following transfection. This level of death induced by DECAY was similar in MCF-7 cells (data not shown), and is similar to the level of death induced by Caspase-3 under similar conditions (Dorstyn et al., 1997). To establish whether DECAY could induce cell death in a Drosophila cell line, SL2 cells were transfected with either full length DECAY or a truncated form, DECAY(MPD), lacking the amino-terminal 21aa residues. Consistent with findings from mammalian cell lines, full length DECAY was
Figure 5.6 Ectopic expression of DECAY in transfected mammalian cells.

(A) Various expression constructs were co-transfected with pEF-β-gal into 293T or NIH-3T3 cells and 24 h post-transfection, cells were fixed and stained with X-gal and were observed for apoptosis. (B) Drosophila SL2 cells were co-transfected with various pRMHa3 vector expression constructs as labelled and pCasper-lacZ. 24 h after transfection cells were heat shocked to induce lacZ expression and then treated with CuSO₄ (0.7mM) for 48 h to induce expression of transfected constructs. Cell survival was quantified by comparing the percentage β-gal positive cells in CuSO₄ treated versus untreated cells.

In both (A) and (B), at least 400 cells were scored for each dish. Bars represent apoptotic or surviving cells ± SEM and was calculated from three independent experiments.
A

![Graph showing % Apoptotic cells for different conditions.]

- Vector
- DECAY
- DECAY C150G
- DECAY (MPD)

B

![Graph showing % Surviving cells for different conditions.]

- Vector
- DECAY
- DECAY C150G
- DECAY (MPD)
- p35
- DIAP1
- DIAP2
- Z-VAD-fmk
- +DECAY (MPD)
unable to induce a significant level of death 48 h after CuSO₄ induction of protein expression (Figure 5.6B). Expression of DECAY(MPD) induced approximately 25% death after 48 h which could be inhibited by co-expression of P35 and to a greater extent by DIAP1, DIAP2 or by treatment with VAD-fmk (Figure 5.6B).

To determine the sub-cellular localisation of DECAY in transfected cells, 293T cells or NIH-3T3 cells were transfected with FLAG-tagged DECAY, and protein was detected by immunofluorescence. The majority of DECAY protein was diffusely present in the cytoplasmic compartment of cells (Figure 5.7A-D). Expression of DECAY or DECAY(C150G) in SL2 cells displayed similar localisation, with most of the protein diffuse throughout the cell (Figure 5.7E-G). Although expression of DECAY(MPD) was weaker, staining was also detected in cytosol but no nuclear staining was seen (Figure 5.7H).

### 5.5 DECAY has a substrate specificity similar to that of the Caspase-3 subfamily

To confirm that DECAY is indeed a caspase, we expressed DECAY(MPD), and the catalytic cysteine mutant (DECAY(C150G)) fused to 6xHis in Eschericia coli. The truncated DECAY(MPD) form was used based on findings from drICE, which is only active once its amino-terminal region is removed (Fraser and Evan 1997). The majority of the protein expressed in E.coli was insoluble and became inactive upon attempts to purify under both native and denaturing conditions. We therefore we analysed caspase activity in the soluble fraction of bacterial extracts using fluorogenic peptide substrates. DECAY did not show significant activity on Caspase-1 substrate YVAD-amc. DECAY(MPD) lysate efficiently cleaved Caspase-3 substrate DEVD-amc (Figure 5.8A). Interestingly, DECAY was substantially more active on the pentapeptide substrate VDVAD-amc, the preferred Caspase-2 substrate. However, in our hands, VDVAD-amc was also cleaved efficiently by Caspase-3. As expected, the DECAY(C150G) mutant did not exhibit any appreciable caspase activity. Likewise, full length DECAY showed only minimal activity on the substrates tested compared to minus prodomain form (data not shown).

We next set out to determine whether DECAY is able to cleave cellular substrates, like its Class II members DCP-1 and drICE. PARP is one of the key cellular substrates of
Figure 5.7 Cellular localisation of DECAY.

Ectopically expressed DECAY localises mainly to the cytoplasmic compartment of transfected cells. (A-B) 293T or (C-D) NIH-3T3 cells were transfected with empty vector (left panels) or DECAY-FLAG (right panels) and 24 h post-transfection, cells were fixed, permeabilised and stained with α-FLAG antibody followed by a mouse IgG-FITC conjugated antibody and cells visualised under fluorescence microscopy.

(E-H) SL2 cells were transfected with empty vector (E); DECAY<sup>C150G</sup>-FLAG (F); DECAY-FLAG (G) or DECAY(MPD)-FLAG (H). 24 h after transfection cells were CuSO<sub>4</sub> induced and left for a further 24 h before fixation and antibody staining as described above.
Figure 5.8 DECAy substrate specificity.

(A) Activity of recombinant DECAy. *E. coli* lysates containing recombinant caspases were incubated with 100μM of various fluorogenic caspase substrates, YVAD-amc, DEVD-amc or VDVAD-amc at 37°C for 30 min and release of -amc was monitored on a fluorimager. Bars represent average fluorescence units ± SEM derived from three independent experiments. (B) Cleavage of truncated PARP protein by DECAy. *In vitro* translated 35S-labelled PARP protein was incubated with recombinant DECAy or Caspase-3 for 3 h at 37°C. Cleavage products were detected by autoradiography. Truncated PARP is translated as a 38 kDa protein which is cleaved at a DEVD site to generate 24 kDa and 14 kDa fragments. As expected the catalytically inactive DECAyC150G mutant does not cleave PARP.
Caspase-3 (Tewari et al., 1995; Nicholson et al., 1995). Being a Caspase-3 like molecule, we assessed whether PARP could serve as a substrate for DECAY in vitro. Incubation of a truncated 35S-labelled PARP protein that contains the Caspase-3 cleavage site was incubated with recombinant DECAY and its cleavage detected by autoradiography. As shown in Figure 5.8B, PARP is efficiently cleaved by DECAY yielding cleavage products identical in size to those generated by Caspase-3 cleavage. This finding suggests DECAY cleaves PARP following the same DEVD sequence as Caspase-3 and further suggests that DECAY has similar substrate cleavage specificity to Caspase-3.

5.6 Processing of DECAY in vitro

To assess whether DECAY could be processed in vitro, 35S-labelled full length DECAY was incubated with various active bacterially expressed caspase lysates and cleavage detected by electrophoresis and autoradiography. As can be seen in Figure 5.9A, DECAY is not processed efficiently by any of the active caspase lysates used. Interestingly, extract prepared from wild type adult flies (w118) and a second instar larval extract were able to induce cleavage of DECAY, indicating the presence of proteases able to mediate DECAY activation during development and also in the adult fly.

As noted in its amino acid sequence, DECAY does not have any conserved aspartate recognition sequences that are optimal target sites for any caspase described. There is a DXXD site (DRDD) at amino acid position #81 and cleavage after this aspartate residue would generate the 26 kDa 'minus-pro-domain' DECAY fragment, and 9 kDa prodomain fragment detected in vitro (Figure 5.9B). The presence of many additional intermediate bands may suggest that DECAY is cleaved following alternative residues rather than aspartate residues. Processing of other Drosophila caspases by a bacterial extract expressing recombinant DECAY could not be detected in our in vitro system (data not shown).
Figure 5.9 In vitro processing of DECAY.

(A) In vitro translated $^{35}$S-labelled DECAY was incubated with Drosophila extracts prepared from larvae, pupae, adult or SL2 cells, or with E. coli extracts expressing various recombinant Drosophila caspases, at 37°C for 3 h. Cleavage products were analysed by protein electrophoresis and autoradiography. (B) Schematic representation of DECAY processing. Putative aspartate sites are indicated to generate fragments of size 26 kDa, 15 kDa and 11 kDa. The presence of intermediate sized bands may be generated by cleavage at alternative sites.
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kDa

35 kDa

26 kDa

9 kDa

15 kDa

11 kDa

B

35 kDa: D34, D98

26 kDa: p15

9 kDa: D34, D98

15 kDa: D34, D98

11 kDa: D34, D98
5.7 DECAY is processed *in vivo* during apoptosis

To assess the processing and activation of DECAY *in vivo*, SL2 cells were transfected with full length DECAY-FLAG and following protein induction, cells were treated with cycloheximide or UV-irradiated, and protein extracts were made for western blot analysis. Interestingly, cycloheximide treatment of cells induced processing of DECAY from its 35 kDa full length protein to 26 kDa and 9 kDa fragments (Figure 5.10). Processing to a 26 kDa fragment could be detected as early as 8 h after treatment and cleavage to the small 9 kDa subunit appeared after 16 h. Processing was not detected under UV-treatment and interestingly there was no marked decrease in DECAY full-length protein following 24 h of apoptotic induction. Since the FLAG-epitope is at the carboxyl-terminus of the protein, the 26 kDa and 9 kDa fragments are likely to represent minus prodomain DECAY and the carboxyl-terminal small subunit.
Figure 5.10 Processing of DECAY during apoptosis.

SL2 cells were transfected with DECAY-FLAG and 16 h after CuSO$_4$ induction, cells were left untreated (A) or where treated with either cycloheximide (25μg/μl) (B) or UV-irradiated (100 J/m$^2$) (C). Cells were harvested at 0, 2, 4, 8, 16 and 24 h for protein, and processing was detected by immunoblotting with α-FLAG antibody.
5.8 Discussion

This final results chapter describes the identification and preliminary characterisation of a new Class II *Drosophila* caspase, DECAY. DECAY is most similar to Caspase-3 like effector caspases and shares similar substrate specificity. Low levels of *decay* transcript are widely expressed during *Drosophila* embryogenesis. Higher expression of *decay* mRNA can be detected in larval salivary gland and midgut which suggests a possible role for DECAY in the programmed deletion of these obsolete tissues during metamorphosis and tissue remodelling. Additionally, moderate expression of *decay* mRNA in nurse cells suggests a possible role for DECAY in nurse cell death following the dumping off cytoplasmic nutrients to developing oocytes. The high levels of *decay* detected in the adult may also suggest a possible important role for DECAY in regulating normal cell turnover and tissue homeostasis in the adult fly. RNA ablation studies did not show any visible defect on the development of the adult fly (data not shown) so ablation of *decay* may be compensated by other caspases. Further analysis is underway to detect whether these flies have any other development defects. Generation of loss-of-function *decay* mutant would also shed light on the role of DECAY in programmed cell death in *Drosophila*.

Mammalian caspases have been proposed to belong to two classes. The upstream initiator, or Class I caspases, and the downstream effector, or Class II caspases. Class II caspases are processed by Class I caspases, and once activated can mediate cleavage of various cellular substrates or of Class I caspases, which possibly serves as a signal amplification mechanism. This has been shown at least for drICE which is activated by DRONC and in turn can process and activate DRONC (chapter 3). As demonstrated in this chapter, we have not been able to demonstrate processing of DECAY by any *Drosophila* or mammalian caspases. However, DECAY can be processed by wild type larvae and adult fly extracts, and also in SL2 cells in response to cycloheximide treatment, suggesting that DECAY activity can be mediated via proteolytic processing by proteins present in these cells and extracts. As noted by other laboratories, the activity of recombinant DCP-1 or DCP-2/DREDD bacterial lysates appears to be consistently low, so we cannot formally conclude that DECAY is not processed by these caspases *in vivo*. 
Further studies are required to delineate the mechanism of DECAY activation. We are currently analysing the regulation of DECAY by other apoptosis regulatory proteins. Both biochemical and genetic interaction studies in the fly should help delineate whether DECAY can be regulated by the H99 gene products or by DIAP1 and DIAP2.

The results presented here demonstrate the cloning of a Class II caspase DECAY that is activated at a late stage during apoptosis, compared to DRONC (Chapter 3), and is able to induce processing of PARP in the execution of cell death. Further studies are required to establish the physiological function of DECAY in apoptosis.
Chapter 6

General Discussion
The identification and characterisation of components involved in the apoptotic pathway are essential to our understanding of the regulation of cell death during development and adult tissue homeostasis. For many years, geneticists have used *D. melanogaster* as an experimental system for the characterisation of gene function in complex signalling pathways. The use of *D. melanogaster* as a model system to study the molecular basis of cell death has become an invaluable tool over the last decade.

At the commencement of this project, the apoptotic pathway in the fly was ill-defined, only three caspases had been identified and their functions in the apoptotic pathway mediated by RPR, HID and GRIM were unclear. However, in the last two years many components of the fly cell death pathway have been characterised including several caspases, DARK and Bcl-2 homologues. The identification of a CED-4/Apaf-1 homologue, DARK, gave rise to the notion of a mitochondrial pathway to apoptosis in the fly that may lead to the activation of caspases in a cytochrome *c* dependent manner (Kanuka *et al.*, 1999b; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999). The identification of a pro-apoptotic Bcl-2 family member, Debc1, was the first indication that *Drosophila* encodes Bcl-2 like regulatory proteins that may function through a DARK-mediated death pathway (Colussi *et al.*, 1999). The primary aim of this study was to identify additional *Drosophila* caspases and analyse their function(s) in apoptosis, to further contribute to an understanding of the cell death pathway in the fly.

Prior to the publication of the *Drosophila* genomic sequence in 2000, we identified two novel caspases, DRONC and DECAY, and have described their characterisation in chapters 3, 4 and 5. Results presented in chapter 3 discuss the identification and initial characterisation of DRONC. The discovery that *dronc* is induced by the steroid hormone ecdysone is the first report of a hormone regulated caspase in metazoans and suggests that upregulation of *dronc* may be crucial to the PCD of larval tissues during insect metamorphosis [chapter 3; (Dorstyn *et al.*, 1999)]. Subsequent studies by Lee and colleagues (2000) have indicated that in *Drosophila* salivary glands, *dronc* transcription is mediated by the ecdysone-induced transcriptional regulator, E93. This was emphasised by the finding that in *E93* mutant flies, ecdysone-induced upregulation of *dronc* is completely
abolished (Lee et al., 2000). In addition to dronc, rpr, hid and dark are also transcriptionally induced by E93 in an ecdysone-mediated response (Baehrecke, 2000; Lee et al., 2000), so transcriptional regulation of these death genes may simultaneously control temporal and spatial cell death in the same developmental PCD pathway. As detailed in chapter 1, ecdysone mediates stage- and cell-specific death during metamorphosis, with the deletion of larval tissues such as midgut and salivary glands, and is involved in the regulation of neurodevelopmental events such as neuronal survival and synaptic remodelling. The induction of caspases by ecdysteroids has recently been shown to result in loss of mitochondrial function and activation of downstream caspases that ultimately lead to structural destruction and PCD of neurons (Hoffman and Weeks, 2000). Ecdysone induction of rpr and grim mediates neuronal cell death in Drosophila but it is not known whether DRONC is involved in this process (Robinow et al., 1997). It will therefore be interesting to analyse whether dronc is upregulated during neural development. It will also be important to analyse whether mammalian caspases can be transcriptionally regulated during development. Such studies are essential to establish the temporal and spatial expression of specific caspases in various developmental events such as neurogenesis and tissue remodelling.

With the completion of the Drosophila genomic sequence, it is clear that DRONC is the only CARD-containing caspase, among the seven caspases in the fly. Given the structural homology shared between DRONC and Caspase-9, it was postulated that DRONC functions in cytochrome c dependent, Apaf-1/Caspase-9-like pathway in the fly. Results presented in chapter 4 of this thesis support this notion on three accounts: 1) DRONC genetically and physically interacts with DARK, the Apaf-1 ortholog in the fly, 2) extracts from dark mutant flies have reduced ability to process DRONC, and 3) Processing of DRONC is enhanced in the presence of cytochrome c and dATP. In mammals, cytochrome c is released from mitochondria during apoptosis and binds to Apaf-1 thereby enabling Apaf-1 oligomerization (Li et al., 1997; Zou et al., 1999). Oligomerized Apaf-1 recruits Caspase-9 and maintains close proximity of Caspase-9 molecules to induce their autoprocessing and activation (Kumar and Colussi 1999; Zou et al., 1999). The role of cytochrome c in Drosophila caspase activation is still unclear.
Like Apaf-1, DARK contains WD40 motifs that mediate cytochrome c binding, but it is not known if cytochrome c binding mediates DARK oligomerization. Studies by Kanuka and colleagues (1999b) demonstrate the requirement of cytochrome c for DARK-induced caspase activation but the question remains as to whether cytochrome c is released from mitochondria in Drosophila cells during apoptosis. Kanuka and colleagues (1999b) have demonstrated release of cytochrome c into cytosol, from mitochondria, during apoptosis mediated by RPR expression or by cytotoxic drugs. However, findings by Varkey and colleagues (1999) suggest this is not the case, but that cytochrome c undergoes a conformational change during apoptosis that mediates binding to DARK, while remaining associated with mitochondria. It is therefore unclear how cytochrome c activates caspases like DRONC and whether DARK can directly interact with cytochrome c in vivo, like the mammalian Apaf-1. In addition, further studies are required to analyse whether DRONC can be recruited to a DARK/cytochrome c/ dATP complex during apoptosis, similar to the Apaf-1/cytochrome c/dATP/Caspase-9 apoptosome that forms in the mammalian system (Li et al., 1997; Srinivasula et al., 1998). Due to the technical difficulties associated with expressing recombinant DARK, we were unable to establish the direct effect of DARK and cytochrome c on DRONC processing and activation. Such studies will be important to understand regulation of caspase activation in the fly.

Results presented in chapter 3 of this thesis demonstrate processing of DRONC in vitro. This event can be mediated by recombinant DRONC(MPD) or drICE, and by extracts prepared from SL2 cells or Drosophila larvae. Subsequent experiments presented in chapter 3 confirmed that DRONC is processed and activated in vivo in SL2 cells in response to various apoptotic stimuli. The mechanism of DRONC processing to generate the cleavage products seen (Figures 3.11 and 3.13) is unclear. The generation of 36 kDa, 18 kDa, and 9 kDa fragments seen in cleavage experiments (chapter 3) represent processing of DRONC at sites additional to aspartate residues. The discovery that DRONC has cleavage specificity for both aspartate and glutamate residues has begun to clarify this issue (Hawkins et al., 2000). This altered cleavage specificity appears to be indicative of the substrate specificity of DRONC. Although the optimal DRONC P1
aspartate tetrapeptide cleavage sequence was found to be TATD, self-cleavage of DRONC was shown to occur at TQTE \textsuperscript{352} site between the large and small subunit boundary but not after DXXD aspartate residues in the prodomain (Hawkins et al., 2000). Consistent with our findings, DRONC was also able to process drICE at a TETD site between the large and small subunit (chapter 3 and Hawkins et al., 2000), so drICE acts as a downstream target of DRONC. This is consistent with studies by Meier and colleagues (2000) who identified DRONC in a yeast-two-hybrid screen through interaction with drICE. The finding that drICE efficiently processes DRONC may serve as an amplification step in the apoptotic pathway. Activated DRONC is able to induce further cleavage of downstream targets (such as drICE itself), which results in the processing of cellular substrates such as lamin Dmo and DREP-1, ultimately leading to cellular demise (chapter 3, Meier et al., 2000).

The importance of DRONC in \textit{Drosophila} developmental PCD has been demonstrated by RNAi studies in chapter 4. Ablation of \textit{dronc} function resulted in a complete inhibition of PCD in embryos. Additionally, the \textit{dronc} dsRNA-injected embryos failed to hatch, suggesting a central role for DRONC as an essential caspase for development and morphogenesis. Development of the nervous system in \textit{dronc} injected embryos appeared normal in our studies and neural differentiation did not appear to be extensively affected. A recent study by Petritsch and colleagues (2000b) suggested that ablation of \textit{dronc} function induces severe hyperplasia of the nervous system. It appears that \textit{dronc} plays a role in the death of neurons and possibly mediates spatial precision of the CNS in the fly. These studies provide a framework for further research into the role of \textit{dronc} during neurogenesis.

A model of the regulation of DRONC in the fly apoptotic pathway is presented in Figure 4.14. The first interesting finding was the demonstration that DRONC-mediated cell death in the fly eye was influenced by the dosage of RPR, HID and GRIM proteins. Consistent with these findings, Hawkins et al. (2000) and Meier and colleagues (2000) demonstrated that RPR, HID and GRIM can mediate death through activation of DRONC.
The finding that GRIM associates with DRONC was the first demonstration that GRIM can induce apoptosis by physically interacting with a caspase (chapter 4). The second important finding was the inhibition of DRONC-induced death by overexpression of DIAP1 in the Drosophila eye (chapter 4, Hawkins et al., 2000; Meier et al., 2000). Additional data in chapter 4 demonstrated physical association between DRONC and DIAP1. Meier and colleagues (2000) further demonstrated direct binding of DIAP1 to the DRONC-CARD. Interestingly, our observations show that DIAP1 can still inhibit DRONC(MPD)-induced death in cell culture, which may be due to the inhibition, by DIAP1, of downstream caspases such as drICE (Hawkins et al., 1999). It is clear that DIAP1 is a key regulator of DRONC activation. Interestingly DIAP1 has been demonstrated to mediate the interaction between DRONC and GRIM (chapter 4). DIAP1 may act by inhibiting GRIM-mediated activation of DRONC. Alternatively, sequestration of DIAP1 by GRIM may induce activation of DRONC. This latter proposal is consistent with the finding that GRIM (and HID) can completely block DIAP1 inhibition of DRONC-induced death of yeast (Hawkins et al., 2000)

In addition, we have also demonstrated interaction between DRONC and DIAP2, which has also been shown to interact with STRICA (Doumanis et al., 2001). It is unclear whether DIAP2 specifically regulates DRONC activation, and whether its inhibitory effect on DRONC-mediated eye ablation is likely to be indirect through interaction with RPR, HID and GRIM (Hay et al., 1995, Vucic et al., 1997, 1998).

In contrast to the data presented in chapters 3 and 4, Hawkins et al. (2000) and Meier et al. (2000) were unable to demonstrate inhibition of DRONC-mediated cell death by P35, suggesting that DRONC is a P35-insensitive caspase. The presence of a P35-insensitive caspase in insects has been endorsed by the demonstration that the activation of Spodoptera frugiperda-Caspase-1 can be blocked by IAP, but not by P35, upon baculovirus infection of lepidopteran cells (LaCount et al., 2000; Manji and Friesen, 2001). In our studies, the inhibition of DRONC-mediated death by P35 is likely to be through the inhibition of downstream Class II caspases such as drICE or DCP-1. As discussed in chapter 4, due the lower level of dronc expression in our studies, the eye
phenotype in our transgenic flies is more sensitive to inhibition of a downstream caspase by P35. Furthermore, the studies by Meier et al. (2000) and Hawkins et al. (2000) were carried out in yeast where no downstream caspases are present for P35 to inhibit, so P35 has no effect on DRONC-induced toxicity. Since DRONC cannot directly bind to, or process P35 (Meier et al., 2000), it is unlikely to be directly inhibited by P35.

The identification of regulators of DRONC is important for our understanding of DRONC function in different developmental events. One approach to identify proteins that regulate, or are regulated by DRONC, is by genetic screens in Drosophila that make use of manipulating the dronc eye phenotype. In such a screen, analysis of suppressors or enhancers of the dronc eye phenotype can be directly visualised, and the regulating gene can then be isolated and characterised. Several candidate proteins have already been isolated through this method (Dr. Shinyop Kim, personal communication). An interesting finding is the interaction detected between DRONC and Numb, a protein involved in the Notch signalling pathway. Petritsch and colleagues (2000a) also identified DRONC as an interacting partner for Numb in a yeast-two-hybrid screen. The Notch signalling pathway is essential for many developmental events, including cell proliferation, differentiation and survival, thereby determining the fate of individual cells (reviewed in Weinmaster, 2000). Activation of Notch receptor through the binding of its ligand, Delta, leads to the proteolytic processing of Notch, which allows it to translocate into the nucleus and directly modulate transcription of various genes. Numb is able to interact with Notch, an event critical for development and survival of neurons during neurogenesis (Artavanis-Tsakonas et al., 1999). Further studies could therefore investigate whether DRONC is involved in regulating apoptosis during neurogenesis. Interestingly, DRONC has recently been shown to induce proteolytic cleavage of Notch during neurogenesis (Petritsch et al., 2000b). This, together with the finding that dronc RNAi effects neural development, suggests that DRONC may be an important molecule in neurogenic events in the fly (Petritsch et al., 2000b). Flies that contain specific mutations or deletions in the dronc gene will be invaluable for analysis of DRONC function during Drosophila development.
Chapter 5 describes the cloning and initial characterisation of a Class II caspase, DECAY. Although little is known about DECAY function in apoptosis, an important feature is that can be processed by adult fly extracts and also in SL2 cells during apoptosis. DECAY shares similar substrate specificity with the caspase-3-like caspases, but to date, the only analysed substrate for DECAY is PARP (chapter 5). It will therefore be of interest to identify other putative substrates of DECAY and determine its importance in apoptosis. RNAi studies did not demonstrate any phenotypic defects caused by ablation of decay function, but the identification of decay deletion mutants should allow us to determine its developmental- or tissue-specific function in PCD. Furthermore genetic interaction studies, similar to those being carried out with DRONC, will be important in determining the function of DECAY in the fly cell death pathways.

In summary, the studies presented in this thesis have described the identification and characterisation of two novel Drosophila caspases, DRONC and DECAY. The work on DRONC has clearly indicated the key role this initiator caspase plays in developmental cell death in Drosophila. Although the role of DECAY is not fully understood at present, the studies presented in this thesis form a basis for future biochemical and genetic investigations of the physiological function of DECAY.
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**Thesis Amendments**

**Chapter 2**
Page 51  
Missing primer sequence used for the amplification of full length DECAY.  
**DECFL:** 5'-**CCCAGCTTGCCATGGACGACACCGACTTC-3'**  
DEC-F primer was used for the amplification of DECAY(MPD).

**Chapter 4**

Figure 4.5  
The interaction between DRONC and GRIM seen in mammalian 293T cells is likely to be mediated by mammalian IAP proteins. Analysis of the interaction with mammalian IAPs should clarify this.

Page 98, 104  
The ternary complex seen between DRONC/GRIM/DIAP1 *in vitro* does not concord with the current mammalian model between Caspase-9/ DIABLO and XIAP. Binding of DIABLO to XIAP displaces Caspase-9, but no ternary complex formation has been detected. Further analysis is required to assess whether a DRONC/GRIM/DIAP1 complex can form *in vivo* and if this interaction is required for DRONC induced apoptosis, and whether DRONC/GRIM and DIAP1 function similarly to their mammalian orthologues.

Figure 4.12  
The asterisks on the right of the figure represent DRONC cleavage products. The 36 kDa band is equivalent to DRONC(MPD) and the 14 kDa band is equivalent to the DRONC small subunit.

Figure 4.14  
RPR/HID/GRIM-induced activation of caspases appears to be through the sequestration and inactivation of DIAP1 activity, thereby enabling activation of DARK and downstream caspases. It is not known whether RPR/HID/GRIM can directly or indirectly activate DARK.

**Bibliography**

Page 126  
Missing reference:  
118: 401-415.