The Identification and Characterisation of Two Novel *Drosophila* Caspases, DRONC and DECAY.

by

Loretta E. Dorstyn, B.Sc (B.Health Sci-Hons)

Enrolled through the Department of Medicine, Faculty of Health Science, University of Adelaide.

Research conducted at the Institute of Medical and Veterinary Science, Department of Haematology, Hanson Centre for Cancer Research, Adelaide.

A thesis submitted for the degree of Doctor of Philosophy, in the faculty of Health Science, University of Adelaide.

March 2001

Contents

Chapter 2: *Materials and Methods*

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.

Acknowledgements

This thesis would not have been possible without supervision from Dr. Sharad Kumar. I would like to take this opportunity to thank Dr. Kumar for allowing me to carry out my PhD studies in his laboratory, for a project that earned me several publications, and for motivation, encouragement and constructive critique of this thesis. Thankyou to Dr. Helena Richardson and Dr. Leonie Quinn, for their invaluable input into this project.

I would also like to thank the University of Adelaide for providing the Australian Post-graduate Research Award that made this study achievable.

Many thanks go to all the members of the Molecular Regulation Laboratory for their enthusiasm, friendship and support.....and for the caffeine fixes at Al's. To Paul, Tash and Kieran for their guidance from when I started. Thanks Tash for all your help with this thesis. To Linda, Kathryn and Stuart for day-to-day discussions and valuable feedback (thanks Linda for the target board.....a great stress relief). Thanks Kathryn for teaching me about flies.

To my close friends Marina, Helen and Karen for their endless friendship and laughter.....thanks Marina and Co. for chats at the 'Swing Cat Club'.

To my family who supported me throughout my many years of study.....a sigh of relief, I will now have a real job. Thanks to mum and dad for inspiration to achieve my goals. To Diana, my sister, my best friend, my editor, my psychologist....thanks for the encouragement.

To all, thankyou.

Publications

Dorstyn L., Kinoshita M. and Kumar S. (1998) Caspases in cell death. In Kumar S. (ed), *Apoptosis: mechanisms and role in disease*, Vol 24: 1-24.

Dorstyn L., Colussi P.A., Quinn L.M., Richardson H. and Kumar S. (1999) DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA*. 96: 4307-4312.

Dorstyn L., Read S.H., Quinn L.M., Richardson H. and Kumar S. (1999) DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. *J. Biol. Chem. 274*: 30778-30783.

Quinn L.M., **Dorstyn L.**, Mills K., Colussi P.A., Chen P., Coombe M., Abrams J., Kumar S. and Richardson H. (2000) An essential role for the caspase DRONC in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem.* 275: 40416-40424.

Harvey N.L., Daish T., Mills K., **Dorstyn L.**, Quinn L.M., Read S.H., Richardson H. and Kumar S. (2001) Characterization of the *Drosophila* caspase, DAMM. *J. Biol. Chem.* In press.

Abbreviations

aa	amino acid
AIF	Apoptosis inducing factor
amc	7-amino-4-methylcoumaride
AO	acridine orange
Apaf-1	Apoptotic protease activating factor
bcl-2	B-cell lymphoma gene
BC-R	Broad complex receptor
BH (1-4)	Bcl-2 homology domains (1-4)
BIR	Baculovirus inhibitor repeats
bp	base pair
BSA	Bovine serum albumin
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
Caspase	cysteinyl aspartate-specific protease
ced	*C. elegans* cell death defective gene
ces	*C. elegans* death specification gene
CrmA	Cytokine response modifier
DAMM	Death associated molecule related to Mch2
DAPAF-1	*Drosophila* Apaf-1 homologue
DARK	*Drosophila* Apaf-1-related killer
DBORG	*Drosophila* Bcl-2 ortholog
DBOK	Drosophila homologue of Bok
DD	death domain
DEBCL	Death executioner Bcl-2 homologue
DECAY	Death executioner caspase related to Apopain/Yama
DED	Death effector domain
DEPC	diethyl pyrocarbonate
DFF	DNA fragmentation factor
DIAP1/2	*Drosophila* inhibitor of apoptosis 1 and 2
DISC	Death inducing signalling complex
DCP-1 /-2	*Drosophila* caspase -1/ -2
DrICE	*Drosophila* ICE
DREDD	Death related Ced-3/Nedd2-like protein
DROB	*Drosophila* ortholog of Bcl-2
DRONC	*Drosophila* Nedd2-like caspase
DTT	1, 4-dithiothreitol
ECL	enhanced chemiluminescence
EcR	Ecdysone receptor
EDTA	ethylenediaminetetra-acetic acid.
egl-1	egg-laying defective gene
EST	expressed sequence tag
FADD	Fas associated death domain
FasL	Fas ligand

FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLIP	FLICE inhibitory protein
fmk	fluoromethyl ketone
GFP	green fluorescent protein
GMR	glass minimal region
GST	glutathione S-transferase
HA	haemaglutinin
HAC-1	Homologue of Apaf-1 / Ced-4
HEPES	N-2-hydroxyethylpiperazine N'2-ethane sulphonic acid
hid	head involution defective gene
HRP	horseradish peroxidase
hsp	heat shock promoter
IAP	Inhibitor of apoptosis proteins
ICAD	Inhibitor of CAD
ICE	Interleukin 1-β converting enzyme
$IgG_{(H/L)}$	immunoglobin G (heavy / light chain)
IP	immunoprecipitation
kb	kilobase
kDa	kilo Dalton
MOPS	3-[N-morpholino] propanesulfonic acid
MPD	minus pro-domain
NAIP	neuronal apoptosis inhibitory protein
nedd2	neural precursor cell expressed developmentally downregulated gene
NP40	Nonidet P40
PARP	poly (ADP-ribose) polymerase
PBS(T)	Phosphate buffered saline (Tween 20)
PCD	programmed cell death
PCR	polymerase chain reaction
RNAi	RNA interference
RT-PCR	reverse-transcribed PCR
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
STRICA	serine / threonine rich caspase
TAE	Tris acetate EDTA
TBST	Tris-buffered saline (Tween 20)
TEMED	N, N, N', N'-tetramethylethylenediamine
TGFR	Transforming growth factor receptor
TNF(R)	tumor necrosis factor (receptor)
TRADD	TNFR associating death domain
TRAF	TNFR associating factor
TUNEL	terminal deoxynucleotidyl transferase-mediated nick end labelling
USP	ultraspiracle
UTR	untranslated region
WB	western blotting

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 the removal of webbing between digits or neural

tube invagination (reviewed in Vaux and Korsmeyer, 1999). 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 **c**ell **d**eath (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* (**e**gg **l**aying **d**efective), *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*.

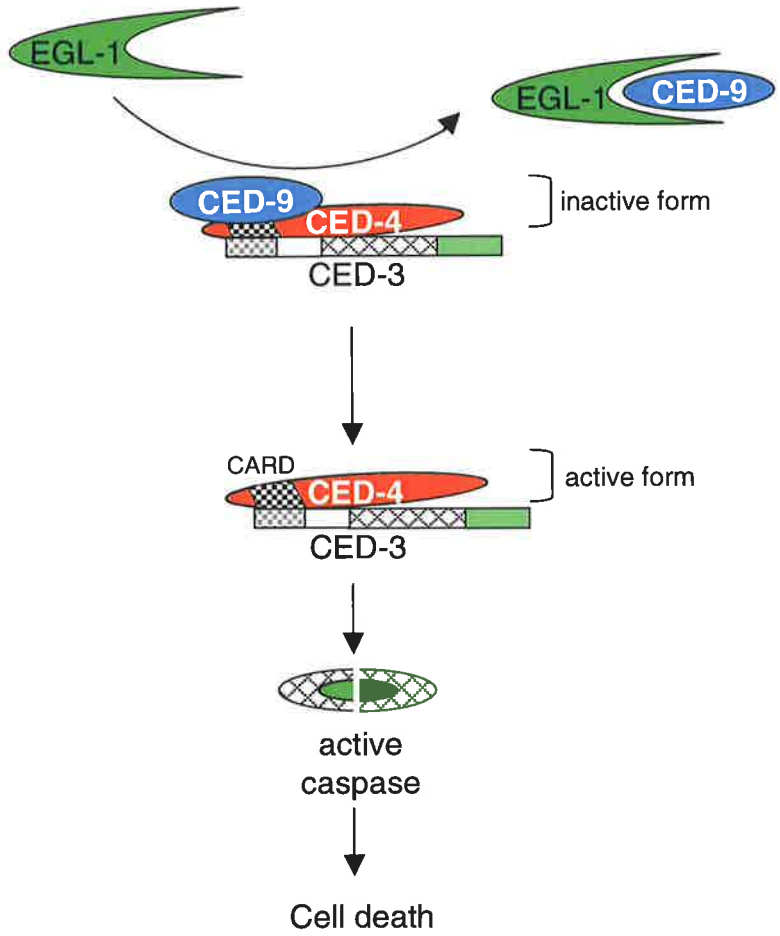
A) Genetic studies in *C.elegans* led to the identification of 15 genes important for PCD. These genes are divided into four groups based on their order of activity. *egl-1, ced-9, ced-4, ced-3* and *ced-8* function in the execution of cell death and the remaining *ced* genes are involved in engulfment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

DECISION TO DIE	EXECUTION	ENGULFMENT	DEGRADATION
ces-2 ⊣ *ces-1*	*egl-1* ⊣ *ced-9*; *ced-4* → *ced-3*; *ced-9* ⊣ *ced-4*; *ced-8*	*ced-1* → *ced-6*; *ced-7* → *ced-6*; *ced-2*; *ced-5*; *ced-10*; *ced-12*	*nuc-1*

B



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/CED-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 constrast 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 **c**ysteinyl **asp**artate-specific prote**ases**, 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 monocytic 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*$^{-/-}$ 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 **n**eural precursor cell **e**xpressed **d**evelopmentally **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 (Fernandes-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 (Fernandes-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.

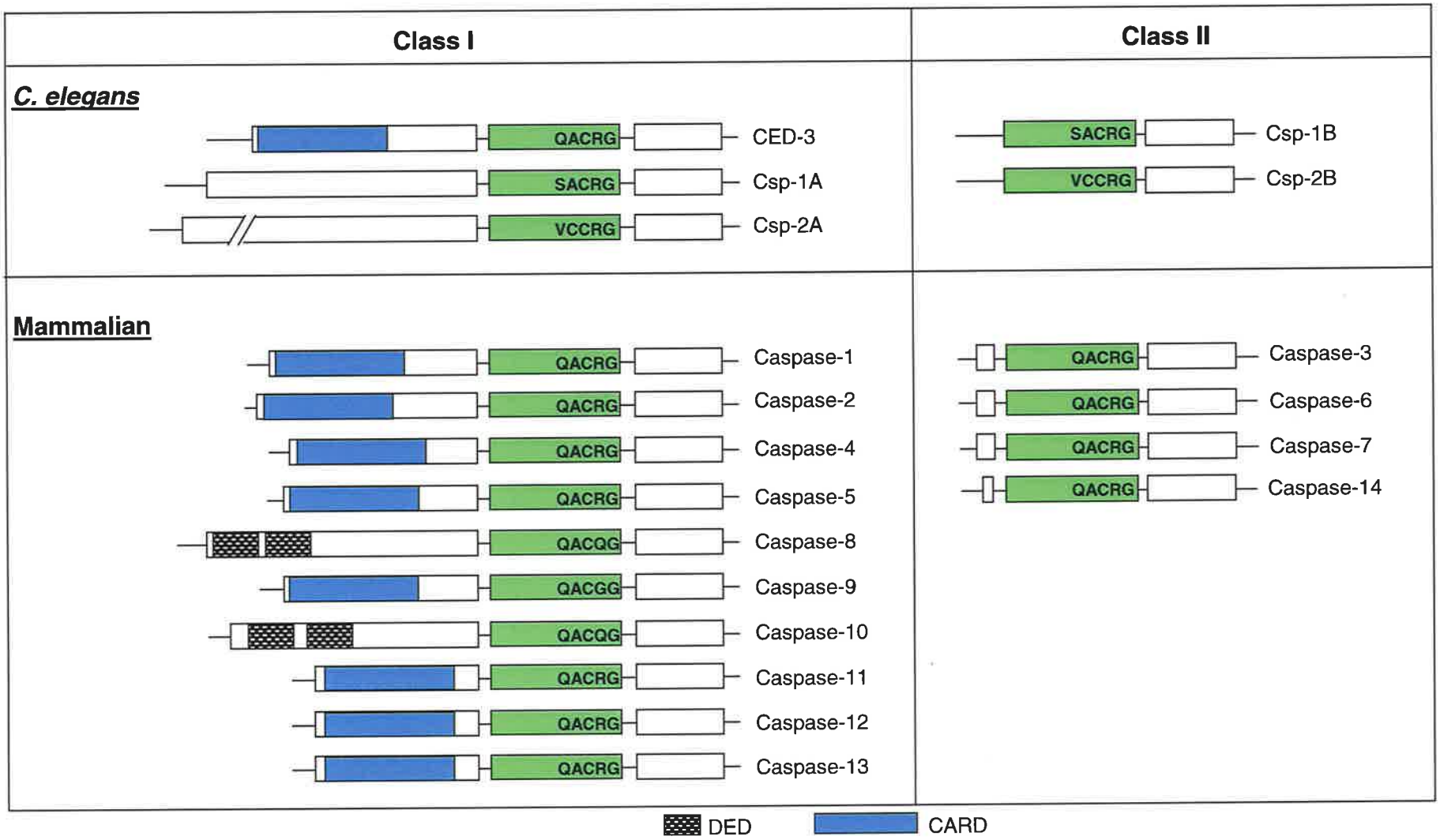
1.3.1 Caspase structure

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.


Class I
Class II
C. elegans
QACRG
CED-3
SACRG
Csp-1A
VCCRG
Csp-2A
SACRG
Csp-1B
VCCRG
Csp-2B
Mammalian
QACRG
Caspase-1
QACRG
Caspase-2
QACRG
Caspase-4
QACRG
Caspase-5
QACQG
Caspase-8
QACGG
Caspase-9
QACQG
Caspase-10
QACRG
Caspase-11
QACRG
Caspase-12
QACRG
Caspase-13
QACRG
Caspase-3
QACRG
Caspase-6
QACRG
Caspase-7
QACRG
Caspase-14
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 (Thornberry 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<u>X</u>D, and that of group III caspases (-6, -8 and -9) (L/V)E<u>X</u>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.

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

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

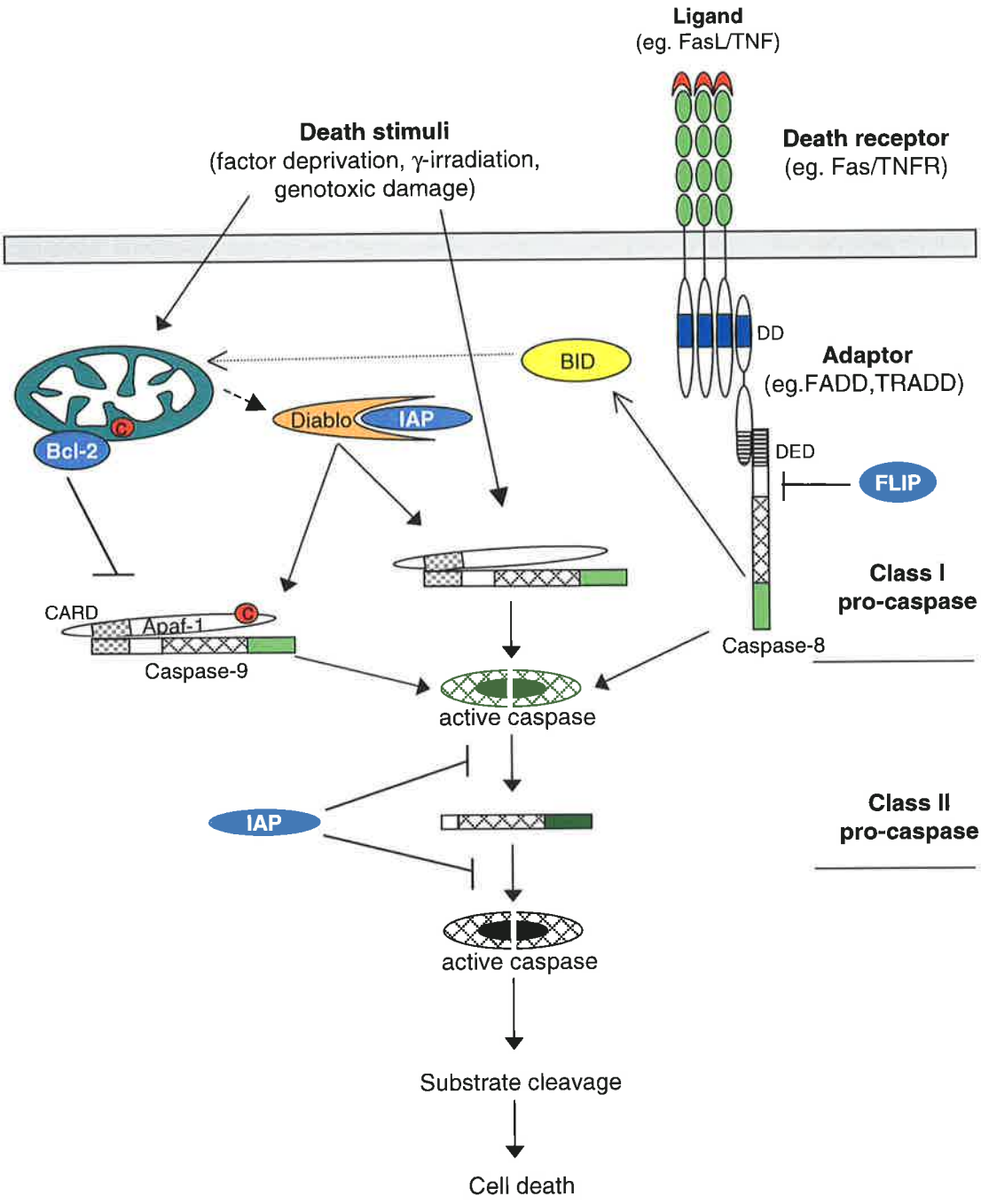
cascades (reviewed in Hengartner, 2000). There are currently two known independent pathways that initiate activation of different Class I caspases and appear to converge to the activation of the same Class II caspases; a receptor-mediated death pathway and a pathway involving cytochrome *c* release from 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.


Ligand
(eg. FasL/TNF)
Death stimuli
(factor deprivation, γ-irradiation,
genotoxic damage)
Death receptor
(eg. Fas/TNFR)
DD
Adaptor
(eg.FADD,TRADD)
BID
Diablo
IAP
Bcl-2
DED
FLIP
Class I
pro-caspase
CARD
Apaf-1
Caspase-9
Caspase-8
active caspase
IAP
Class II
pro-caspase
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 ($\Delta\Psi m$), 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 $Bclx_L$ 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.*,

1992). 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.

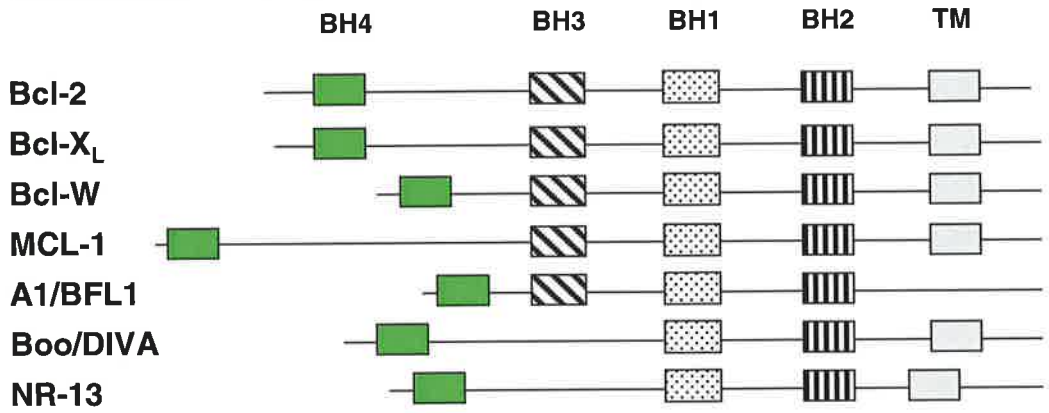
1.5.2 Bcl-2 family of proteins.

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

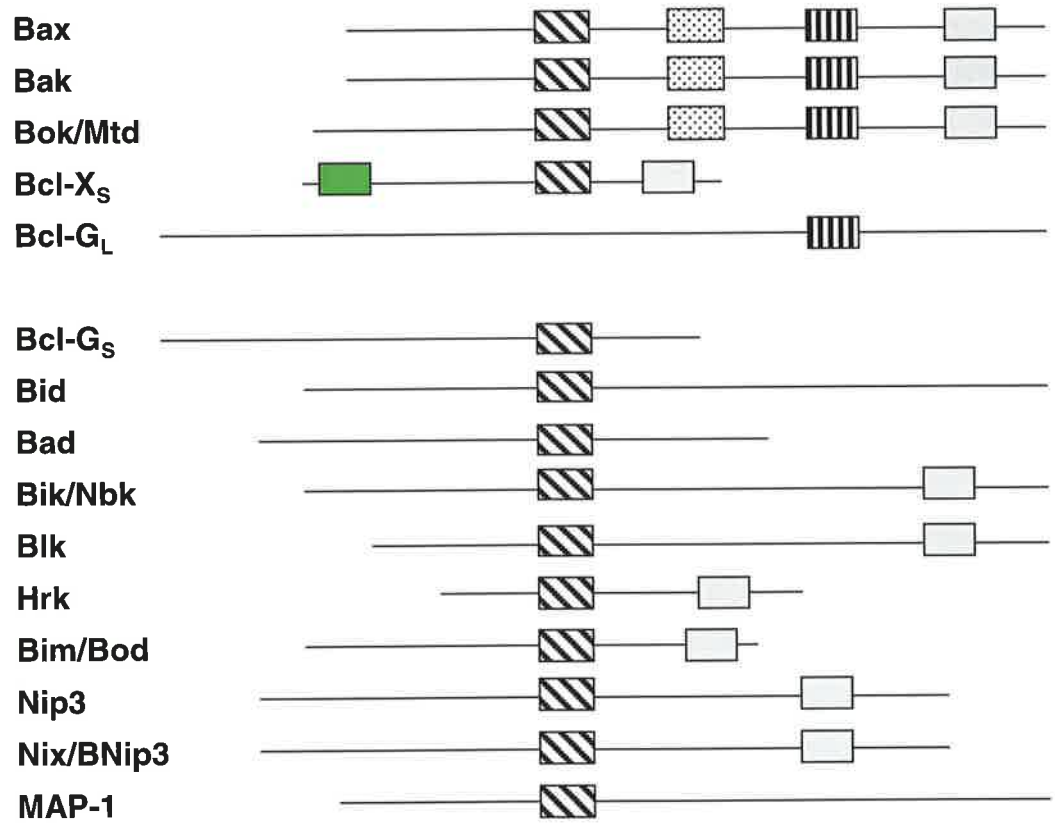
Figure 1.4 The Bcl-2 family.

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)

ANTI-APOPTOTIC



PRO-APOPTOTIC



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-x_L 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-x_S, 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 (Oltavi *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-x_L 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-x_L (reviewed in Gross *et al.*, 1999; and Baliga *et al.*, 2001). It has been proposed that Bcl-2 and Bcl-x_L 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-x_L (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-x_L 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-x_L. 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-x_L* 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 *bax* is also associated with human gastrointestinal cancers and some leukaemia's (Rampino *et al.*, 1997) and loss of *bcl-2* is associated with the neurodegenerative disorder spinal muscular atrophy (SMA) (Iwahashi *et al.*, 1997). *Bax* is also upregulated in response to ischaemic injury which is concomitant with a down regulation in *bcl-2* and *bcl-x*$_L$ (Krajewski *et al.*, 1995). *Bak* knockout mice are essentially normal but when both *bak* and *bax* genes are simultaneously deleted, the effect is deleterious with only 10% survival rate. *Bak*$^{-/-}$ / *bax*$^{-/-}$ 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 *et al.*, 2000). Together these findings emphasise the importance of Bcl-2 family of proteins in apoptotic regulation.

1.5.3 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 *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 *et al.*, 1996). A family of mammalian IAP proteins was subsequently identified based on their homology to baculovirus IAP and consists of xIAP (MIHA/hILP), cIAP1 (MIHB), cIAP2 (MIHC) and Survivin (Duckett *et al.*, 1996; Uren *et al.*, 1996; Liston *et al.*, 1996; Tamm *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 *et al.*, 1998a, 1999). Expression of xIAP and cIAP1 can block apoptosis induced by the overexpression of caspases and act through direct binding and inhibition of caspases (Uren *et al.*, 1996; Deveraux *et al.*, 1997; Dorstyn and Kumar 1997; Roy *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).

Viral

OpIAP

CpIAP

C.elegans

BIR1

BIR2

Mammalian

c-IAP1

c-IAP2

XIAP

pIAP

NAIP

mBRUCE

Survivin

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 β*FTZ-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 Baehrecke, 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/$Bclx_L$, 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, *Sf*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* (***Dros**ophila* **Cas**pase-**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 GRIM-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 *dredd* 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 I*k*B-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 IκB 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 IκB 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 (**D**eath **A**ssociated **M**olecule related to **M**ch2) 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 γ-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 **ca**spase comprising a **s**erine/threonine **ri**ch 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 QAC**Q**G site, DCP-2 contains a QAC**QE** catalytic active site, where as both DAMM and STRICA contain a QAC**K**G 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 Dm_O, 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).

Caspase Class	Caspase members	Substrate specificity	Substrates
Class I	DCP-2 STRICA		Relish
Class II	DCP-1 drICE DAMM	DEXD, TETD DEXD, DHTDA	drICE, PARP, lamin Dm_o, P35 dICAD, PARP, lamin Dm_o, P35

Table 1.2 *Drosophila* caspase classes and substrate specificity. *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

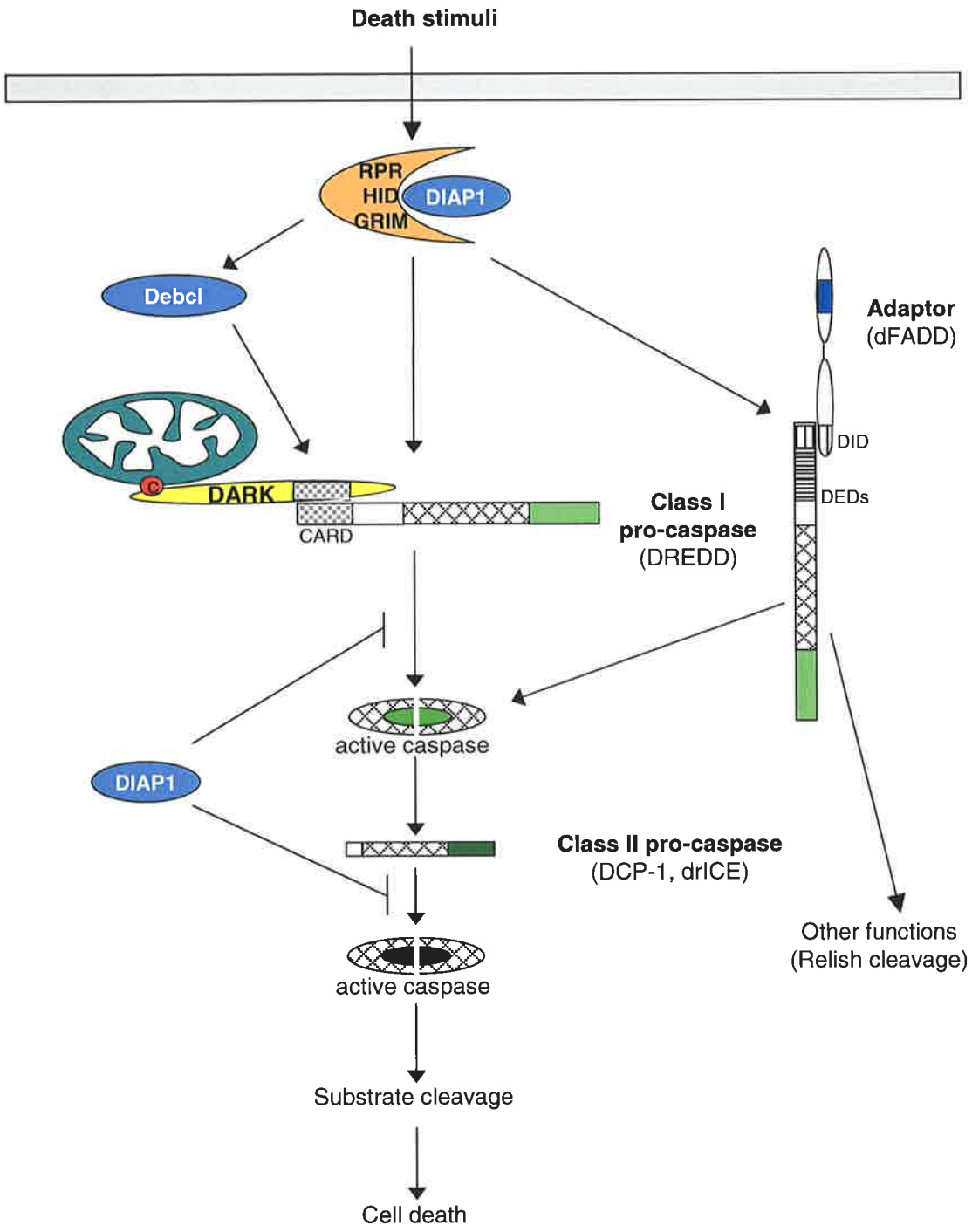
The regulation of *Drosophila* caspases has only been established in the last year with the cloning of Apaf-1 and Bcl-2-like proteins in the fly. A proposed model for the regulation of caspases in the fly apoptotic pathway is displayed in Figure 1.6.

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

Figure 1.6 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
DIAP1
Debcl
Adaptor
(dFADD)
DID
DEDs
DARK
CARD
Class I
pro-caspase
(DREDD)
active caspase
DIAP1
Class II pro-caspase
(DCP-1, drICE)
Other functions
(Relish cleavage)
active caspase
Substrate cleavage
Cell death

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 *diap1* 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 *diap1* or *diap2* can suppress death due to over-expression of *rpr*, *grim* or *hid*. Zygotic loss-of-function *diap1* 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 $O.D_{260nm}$ of 1.0 represents 50μg/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 ¾ down the gel. DNA was visualised by staining the gels in ethidium bromide solution (2μg/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 *EcoRI* digested fragments of bacteriophage SPP1 and *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 10-50μl total volume comprising DNA, 1 unit restriction enzyme/μg 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_2O for 1-3 h at 37°C. Reactions were terminated by either heat inactivation at 65°C or 85°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 H_2O 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/choroform extraction

DNA solution was made up to at least 200µl and an equal volume of phenol/choroform (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 H_2O. 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 H_2O 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 $MgCl_2$, 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-tryptone [Difco], 5g/L Bacto-yeast extract [Difco], 5g/L $MgSO_4$, and pH7.6 with KOH) was innoculated 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 ψb 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 6000rpm, 5 mins, 4°C. Medium was completely aspirated and cells resuspended in 40ml cold Tfb I (30mM KOAc, 100mM KCl, 10mM $CaCl_2.2H_2O$, 50mM $MnCl_2.4H_2O$, 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 $CaCl_2.2H_2O$, 10mM KCl, 15% Glycerol adjusted to pH 6.5 with 0.5M KOH and filter sterilised). Incubated cells on ice for 15 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 $MgCl_2$, 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 α-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 H_2O.

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 H_2O. 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 H_2O. 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

$1x10^7$ cells were washed twice in PBS and pelleted at 1500g, 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 H_2O. 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 H_2O, 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/m^2.

2.5.3 Membrane hybridisation and blotting

a) Preparation of ^{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 α^{32}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 Denhardts 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* ATGCAGCCGCCGGAGC-3'

Dr-R 5'-*CGGGAATTC* CTATTCGTTGAAAAACCCGG-3'

Dr-F_{HA}	5'-*CCAAGCTT*ATG*TACCCATACGACGTCCCAGACTACGCT*CAGCCGCCGGAGCTCAT-3'
Dr-R_{HIS6}	5'-*CTAGTCTAGA*TTA*ATGATGATGATGATGATG* TTCGTTGAAAAACCCGGGAT-3'
Dr-601	5'-AAGATCACCCAGCGTGGTC-3'
Dr-1015	5'-GCCGAAAAGGACAGCAAG-3'
Dr-R_{GFP}	5'-*CGGGATCCCG* TTCGTTGAAAAACCCGGGAT-3'
Dr-F^{C318G}	5'-ATGTTTCCCTTTGGCCGCGGCGATG-3'
Dr-R^{C318G}	5'-CATCGCCGCGGCCAAAGGGAAACAT-3'
DrMPD-F	5'-*GGAATTCCATATG*TCAAGGCCACCCTTTATCTCG-3'
DrP14-F	5'-*GGAATTCCATATG*TTGGGCCATCCAAAGAATCAA-3'
DEC-F	5'-*CCCAAGCTT* ATGCCACCAAGATCCCATA-3'
DEC-R_{FLAG}	5'-*CCGGAATTC* TCA*CTTGTCATCGTCGTCCTTGTAGTC* GGTCTTGGGCTTAACACG-3'
DEC-F(koz)	5'-*GGCGGATCCGCCGCCATGG*CACCAAGATCCCATACG-3'
DEC-R_{HIS6}	5'-*CCGGAATTCTCAGTGGTGGTGGTGGTGGTG* GGTCTTGGGCTTAACACGCAG-3'
DEC-F^{C150G}	5'-CATCCAGGCCGGTCGCGGAGCCA-3'
DEC-R^{C150G}	5'-TGGCTCCGCGACCGGCCTGGATG-3'
DEC-180	5'-TGCAGCGTCGCCTCCAT-3'
DEC-433	5'-TCTTCATCCAGGCCTGTCG-3'
DEC-R_{GFP}	5'-*GGCGGATCC*CGGGTCTTGGGCTTAACACGCAG-3'
DEC-FpET	5'-*GCGAATTCCATATG*CCACCAAGATCCCAT-3'
DEC-783	5'-CTCAACCAGATGAAGGAAAT-3'
T7	5'-GTAATACGACTCACTATAGGGC-3'
Sp6	5'-GATTTAGGTGACACTATAG-3'
PRM-F	5'-AATGTGCATCAGTTGTGG-3'
PRM-R	5'-TTATTATCGTATTAGGAGAA-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:

GAATTC – EcoRI

GGATCC – BamHI

AAGCTT – HindIII

CATATG – NcoI

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 H_2O. Primer concentration was determined by spectrophotometry at absorbance of 260nm (OD_{260} 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 H_2O.

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 $MgCl_2$, 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 H_2O. 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 $(NH_4)_2$ SO_4, 2mM $MgCl_2$, 0.1% Triton X-100, 0.1mg/ml BSA) and 2.5 units Pfu polymerase in a total volume of 50µl sterile H_20. 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 $(NH_4)_2SO_4$, 2mM $MgSO_4$), 2mM dNTP mix, 100ng each forward and reverse primers and 2.5 units Pwo polymerase in sterile H_2O. 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 $(NH_4)SO_4$, 20mM Tris-HCl pH 8.8, 2mM $MgSO_4$, 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 H_2O. 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 *Dpn*I 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, $MgCl_2$, thermal stable pyrophosphatase, AmpliTaq polymerase) and the volume was made up to 20 µl with sterile H_2O. 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^{TM} 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^{C150G}: DECAY cysteine mutant (C150G) generated by Quikchange^{TM} mutagenesis using pOT2-DECAY as template DNA and DEC-F^{C150G}/R^{C150G} 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:$CHCl_3$ 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-RPR$_{FLAG}$, pcDNA3-GRIM$_{FLAG}$, pcDNA3-HID$_{FLAG}$. Constructs kindly provided by Dr Vishva Dixit (Genentech, San Fransisco, USA). Each encode a C-terminal FLAG-tag

pcDNA3-DCP1$_{HA}$: *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-DCP2$_{HA}$: *dcp-2* cDNA was amplified by PCR from pT7-DREDD plasmid (provided by Dr John Abrahms). Primers were flanked with EcoRI/XbaI sites and a 3' HA-tag sequence and PCR product was cloned directionally into pcDNA3.

pcDNA3-drICE$_{HA}$: *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-DIAP1$_{MYC}$: DIAP1 cDNA was released from pBs-DIAP1$_{MYC}$ (provided by Dr Bruce Hay) with EcoRI/BamHI and cloned directionally into pcDNA3.

pcDNA3-DIAP2$_{HA}$: 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^{C318G}/ DRONC(MPD): DRONC (wild type, C318G mutant and minus prodomain) fragments amplified by PCR and ligated blunt into pRMHa3.

pRM-DECAY/mDECAY^{C150G}/ 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$_{HA}$/DCP2$_{HA}$: 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 $_{HA}$: 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 $_{HA}$: DIAP1 cDNA was HA-tagged by PCR amplification from pBs-DIAP1$_{MYC}$. PCR product was digested with EcoRI/BamHI and cloned directionally into pRMHa3.

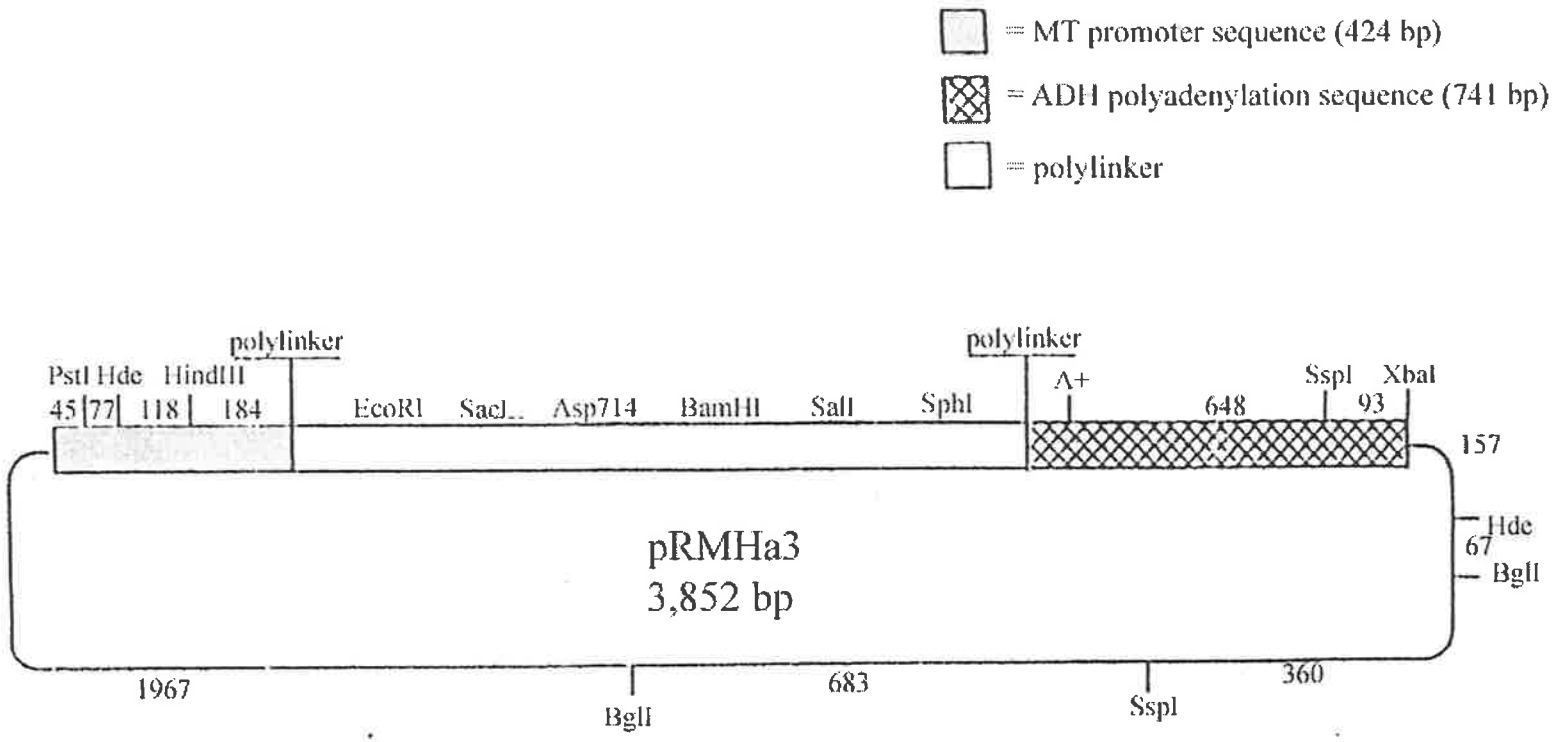


Figure 2.1 pRMHa3 *Drosophila* expression vector. Expression of inserts are driven by the metallothionein promoter. The 3' end of Drosophila ADH gene and polyadenylation site follows the polylinker.

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-p35$_{HA}$: 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-RPR$_{FLAG}$/GRIM$_{FLAG}$: Reaper-FLAG and Grim-FLAG fragments were digested out of pcDNA3 with BamHI/XhoI and cloned into the BamHI/SalI sites of pRMHa3.

pRM-HID$_{FLAG}$: 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-DARK$_{MYC}$, pRM-DARK$^{1-411}{}_{MYC}$, pRM-DARK$^{91-411}{}_{MYC}$: Kindly provided by Dr John Abrahms.

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

pUAST-DRONC/**m**DRONC^{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/**m**DECAY^{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).

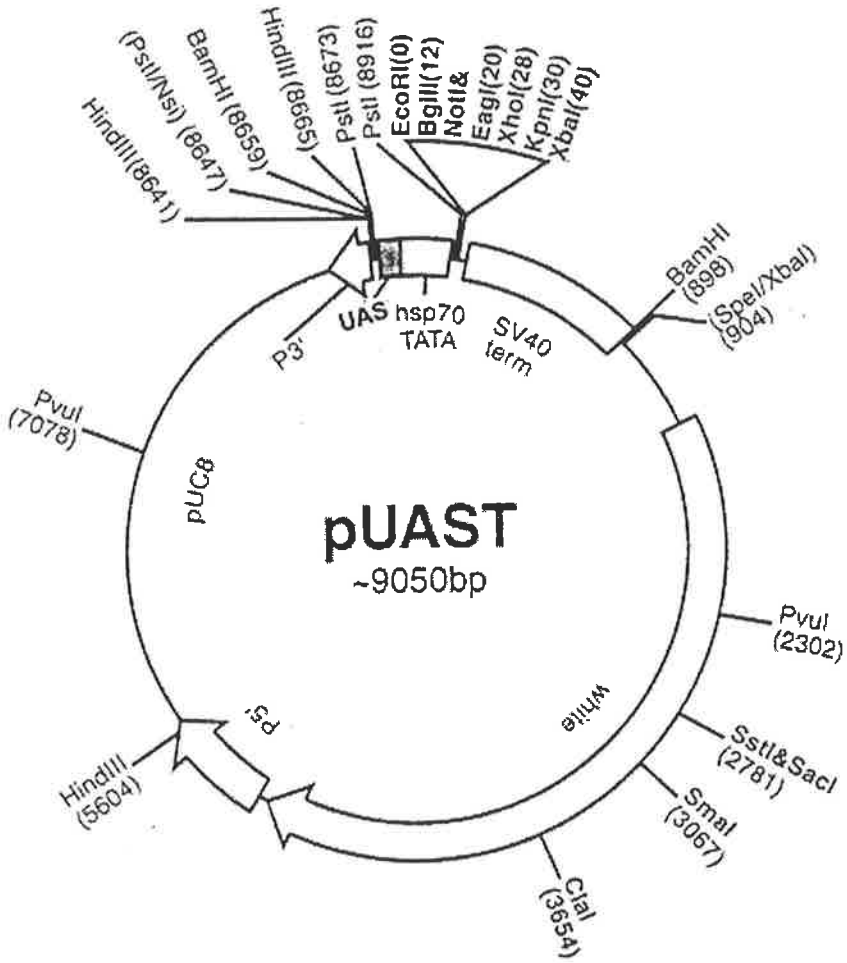


Figure 2.2 pUAST transgenic expression vector. pUAST is a transposable P-element vector and contains five yeast UAS, GAL4 binding sites. Target genes are cloned into a polylinker following the UAS promoter region and *hsp70* TATA box. (adapted from Sullivan *et al.*, 2000)

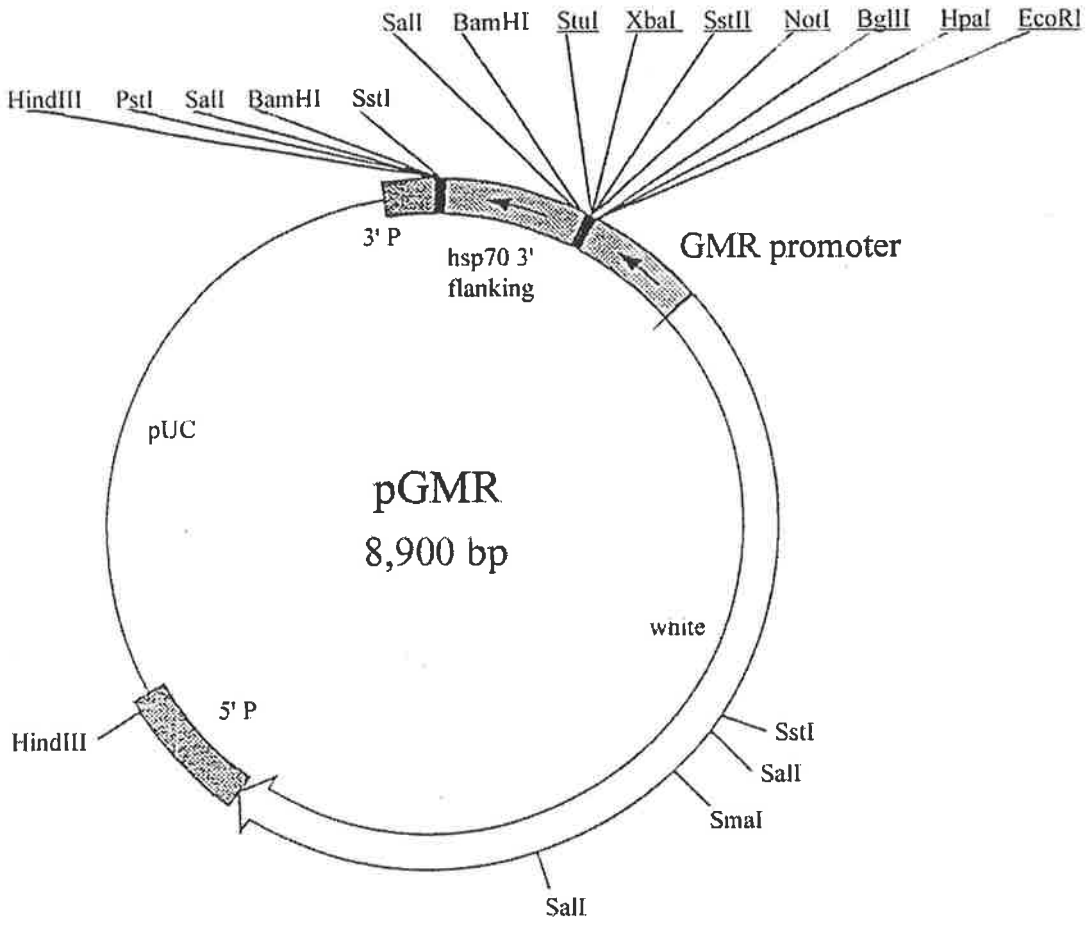


Figure 2.3 pGMR expresion vector. Expression of inserts are driven by the *Drosophila* eye specific, glass minimal region (GMR) promoter.

2.7.4. Bacterial expression constructs

pET32b-DRONC/**m**DRONC^{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/**m**DRONC^{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/**m**DECAY^{C150G}: Wild type and C150G mutant DECAY were PCR amplified using DEC-F_{GFP}/DEC-R_{pET} primers. PCR product was digested with NcoI/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 40μg/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 (BIOTECX 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_2O.

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% xyelene 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 NaH_2PO_4, 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/m^2. Membranes were pre-hybridised at 42°C for 4-5 h, in 10ml RNA hybridisation buffer (50% formamide, 5xSSPE, 1mM EDTA pH8, 5x Denhardts 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 digoxygenin-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 $NaPO_4$ pH7.8, 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 Bicinchoinate (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^{1118}), $dark^{CD4}$ or $dark^{CD8}$ 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 $1x10^7$ 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 ^{35}S-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 ^{35}S-methionine, 1µl RNasin Ribonuclease inhibitor, 1µg DNA template and sterile H_2O. 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 ^{35}S-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 ^{35}S-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 polyvinylidine 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/ or 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 H_2O 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 pH7, 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 ^{35}S-DRONC and / or ^{35}S-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

$5x10^5$ 293T cells or $3x10^6$ 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 polyvinylidine 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 H_2O:MeOH:acetic acid, 5:4:1). Gels were stained for at least 1 h, followed by destaining in several changes of H_2O: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.

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 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 Na_3PO4, Na_2HPO4, 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 = OD_{280} 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% CO_2.

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 $75cm^2$ 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 25cm^2 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 with 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 $MgCl_2$ 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, $2x10^6$ 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.hslacZ reporter plasmid. Where indicated plasmid DNA was co-transfected with pMT-p35, DIAP1, DIAP2 or $DRONC^{C318G}$ 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 800ul 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

times. Following heat shock, cells were split into halves in two 35mm dishes, one dish treated with 0.7mM $CuSO_4$. Where indicated, 50μM z-VAD-fmk (Enzyme Systems Inc) was added to cells at the time of $CuSO_4$ addition. 48 h after $CuSO_4$ induction, cells were stained for β-galactosidase and % surviving cells calculated as the % β-gal positive cells in $CuSO_4$ 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 $CuSO_4$ for 16 h, before treatment with etoposide (Calbiochem) at 40μM, UV irradiation (50-100 Joules/m^2) 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 $CuSO_4$ 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 than 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 CO_2 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*s 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 fir *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-dronc*^{C318G}*GFP* 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*^{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*^{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 (w^{1118}) flies; to *GMR-p35, GMR-diap1, GMR-diap2* expressing flies; to strains containing a deficiency of *rpr, hid* and *grim [Df(3L)H99], diap1 [Df(3L)brm11* and *Df(3L)stf-13],* or *diap2 [Df(2R)Jp1]*; to a specific loss of function allele of *diap1* [*thread*^{5}]; or crossed to P allele mutations of *dark* or *dark*^{CD4}, *dark*^{CD8} and *l(2)k11502* hypomorphic alleles. To test the interaction of a *dronc* deficiency *Df(3L)AC1* at 67A2-67D13) with *GMR-hid* or *GMR-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 *l(2)k11502* stocks were obtained from the Bloomington Stock Center.

2.12.9 Immunohistochemistry

Antibody staining of *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, Pasedena, 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% parafrmaldehyde 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 thee 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 (***Dro**sophila* **N**edd-2 like **c**aspase). 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.

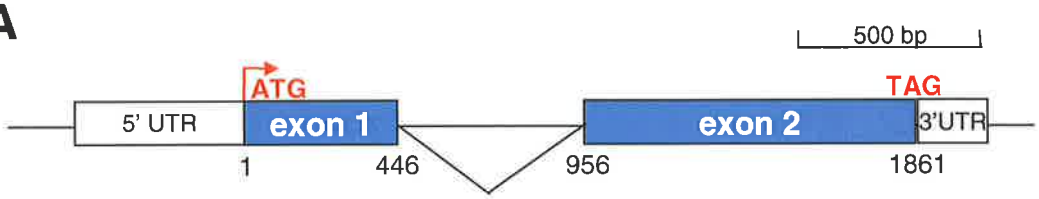
The putative DRONC protein consists of 450 amino acid residues (Figure 3.2A). *In vitro* translation of mRNA generated from *dronc* cDNA produced a 50 kDa protein consistent with the expected size (Figure 3.2B). A unique feature of DRONC is its PFCRG active site, encompassing the catalytic cysteine (Cys318) 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). The

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



B

```
   1 tttggcggcg cgaaacgctc gttggtcctt ttgtttcgaa agaaatccta ttcgaagatc
  61 cccgatcctt gctaggatca tctagtgcaa tatatagact agttaattta cttttggaaa
 121 aataaggaca ccagcagggc cgccgatttg tgccccttc ttgaaagtcg caaaacaaaa
 181 acaacgacga caacaacaaa gcggagacaa agaatcgaca agtagcgata aacgaaatca
 241 ttcccgggaa aaccttggag acgggtgatt cactgccaat accactgcca attggagact
 301 gatcacggca gccatccttg gcgcgcccaa taagcggagt caccggaacg cgtggaagcc
 361 atatccggaa tgcagccgcc ggagctcgag attggaatgc cgaagaggca tcgcgagcat
 421 atacgcaaga atctgaatat actggttgaa tggacgaact acgagcgtct ggccatggag
 481 tgcgtgcaac agggcatcct aaccgttcag atgctaagaa atacgcagga tctcaatggc
 541 aagccattca acatggacga gaaggatgtg cgtgtggagc agcatcgtag gctcctattg
 601 aagatcaccc agcgtggtcc caccgcctat aacctgctga tcaatgcact gcgcaatatc
 661 aattgtctgg atgcggccgt tctattggaa tccgtcgatg agtccgattc aaggccaccc
 721 tttatctcgc taaacgaacg gagaaccagc cggaagtcgg ccgatattgt ggacacaccc
 781 tcacccgaag cctccgaagg accctgcgtt agcaagctcc ggaatgagcc gctgggagca
 841 ctcacccctt atgtgggtgt cgttgacggt cccgaggtaa aaaaatcgaa aaagatacat
 901 ggtggggata gtgccatatt gggcacatat aagatgcaat cacgtttcaa ccgaggcgtt
 961 ttgctaatgg ttaacataat ggactatccg gatcaaaacc gtcgacggat cggagccgaa
1021 aaggacagca agtcgttgat acacttgttt caagaactga attttacgat tttcccctat
1081 gggaacgtga atcaggatca gttctttaaa cttctgacaa tggtgacctc ctcgtcgtat
1141 gtgcagaata ccgagtgttt cgtaatggta ctgatgacac acggcaacag tgtggaggga
1201 aaagagaagg tggagttttg cgatggatct gtggtcgata tgcagaagat caaggaccat
1261 ttccagacgg ccaaatgtcc ttatttggtg aacaagccga aggtgcttat gtttcccttt
1321 tgccgcggcg atgaatatga tttgggccat ccaaagaatc aaggcaatct catggagcca
1381 gtgtatacgg cgcaagagga gaagtggcct gacacccaga cggagggcat acccagcccg
1441 agcaccaatg tgccaagtct tgccgacact ctggtctgct atgctaatac gccgggctat
1501 gttacccacc gcgatctcga cacgggcagc tggtacatcc agaagttttg ccaagtgatg
1561 gccgatcatg cccacgacac agaccttgag gatatcctaa agaagacgag cgaagccgtg
1621 ggtaataagc gcaccaagaa gggttccatg cagacaggtg cctatgataa tcttggcttt
1681 aataagaaac tctacttcaa tcccgggttt ttcaacgaat agttgccgcc actggacatt
1741 ttatcattcc ggatgcattt ttaaccgcat ttatgttctt atcgtcgcat ttagtatgtc
1801 ttttagatta tgtgttctgt gctcgcgtgc tataaaatgt ttatattgta acaaatactc
1861 attcaagtat tcttctaata cgtattgcat acctcattta aaggtacgaa attatgtaat
1921 ttatgtgata actgccagaa atctacaaat attataatac agagttccag tatatacaca
1981 gccagatttt tgaaatcgaa catttaggca aaatgtaaag caattttcca gtctgatttt
2041 tgagtgtgta cacttacaaa ataaacgatg atatttcaca acaaaccgca gtttgtatat
2101 aattaaaatc tatttacatt tctaaaattt cattgggaca ctaattatta tataatat
```

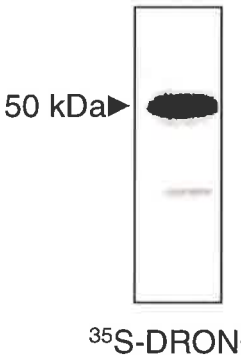
Figure 3.2 Primary structure of DRONC protein.

(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.

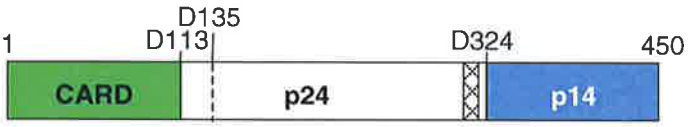
A

```
MQPPELEIGM PKRHREHIRK NLNILVEWTN YERLAMECVQ QGILTVQMLR NTQDLNGKPF  60
NMDEKDVRVE QHRRLLLKIT QRGPTAYNLL INALRNINCL DAAVLLESVD ESDSRPPFIS 120
LNERRTSRKS ADIVDTPSPE ASEGPCVSKL RNEPLGALTP YVGVVDGPEV KKSKKIHGGD 180
SAILGTYKMQ SRFNRGVLLM VNIMDYPDQN RRRIGAEKDS KSLIHLFQEL NFTIFPYGNV 240
NQDQFFKLLT MVTSSSYVQN TECFVMVLMT HGNSVEGKEK VEFCDGSVVD MQKIKDHFQT 300
AKCPYLVNKP KVLMFPFCRG DEYDLGHPKN QGNLMEPVYT AQEEKWPDTQ TEGIPSPSTN 360
VPSLADTLVC YANTPGYVTH RDLDTGSWYI QKFCQVMADH AHDTDLEDIL KKTSEAVGNK 420
RTKKGSMQTG AYDNLGFNKK LYFNPGFFNE                                   450
```

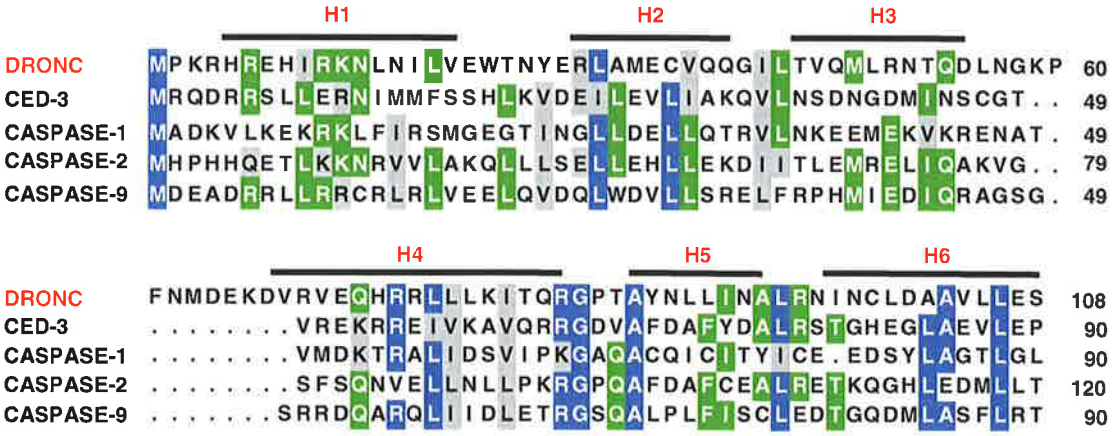
B



C



D



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.

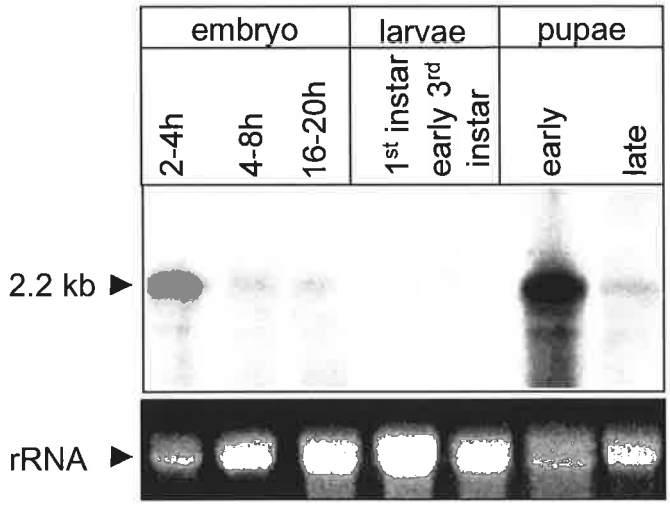
Consistent with Northern data and RT-PCR, *in situ* hybridisation analysis of *dronc* mRNA demonstrated high expression in stage 1-4 syncitial embryos (Figure 3.4). Zygotic expression does not begin before stage 5, so this early expression of *dronc* represents maternally derived mRNA. *dronc* mRNA is 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

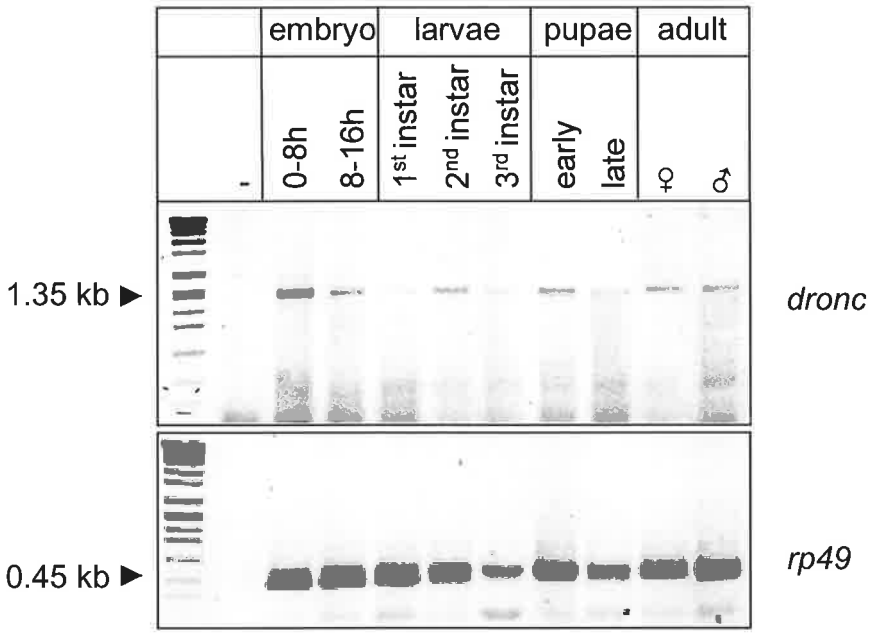
Figure 3.3 Expression of *dronc* mRNA during *Drosophila* development.

(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 potion 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.

A


embryo
larvae
pupae
2-4h
4-8h
16-20h
1st instar
early 3rd instar
early
late
2.2 kb ▶
rRNA ▶

B


embryo
larvae
pupae
adult
-
0-8h
8-16h
1st instar
2nd instar
3rd instar
early
late
♀
♂
1.35 kb ▶
dronc
0.45 kb ▶
rp49

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.

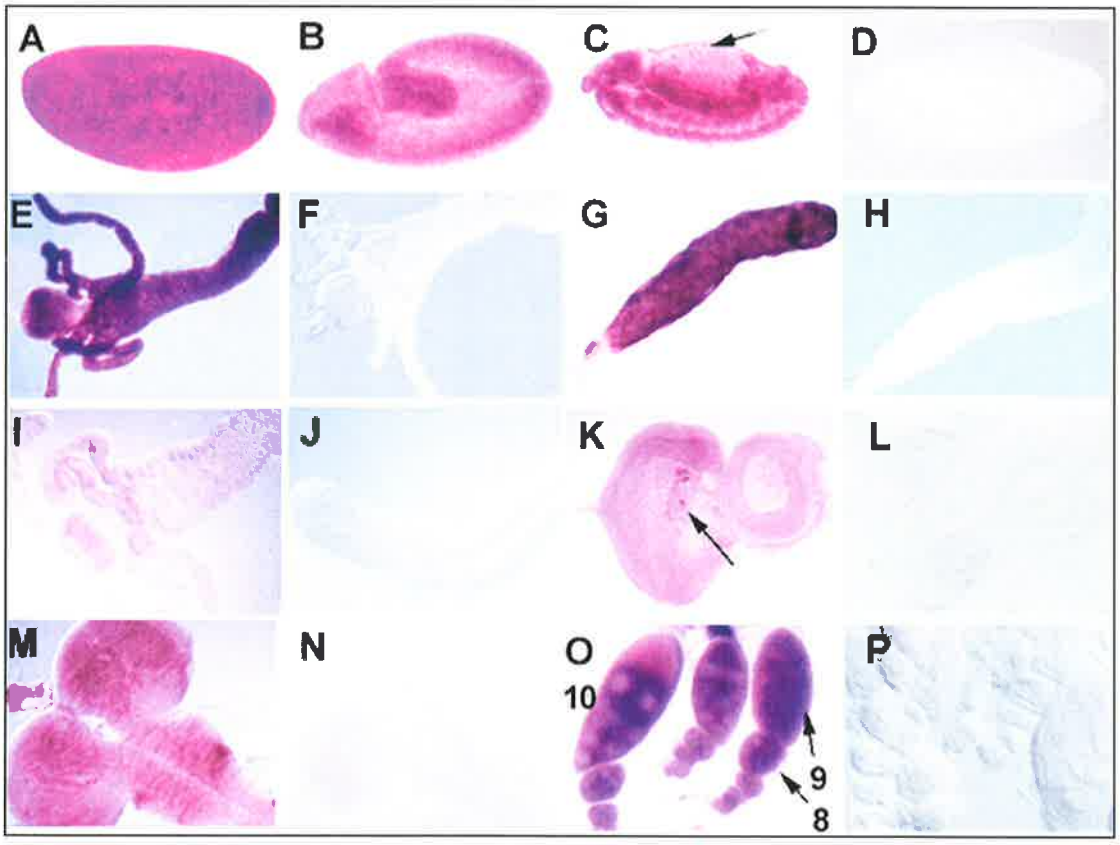
(L) *dronc* sense control probe on eye imaginal disc.

(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.


A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
10
9
8
P

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.

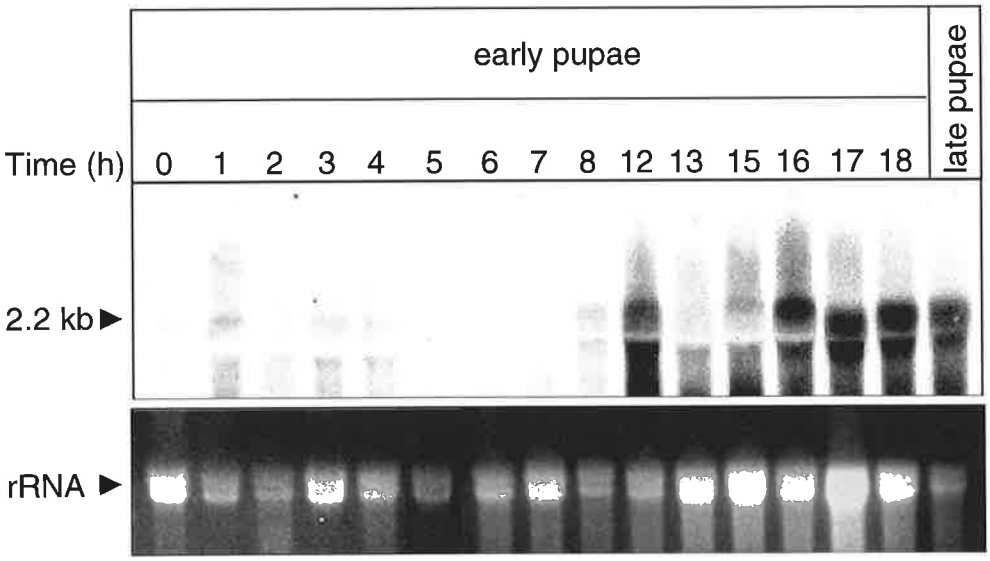
The high expression of *dronc* mRNA during early pupae stages in the midgut and salivary gland prompted the idea that DRONC may be a mediator of apoptosis of these tissues during metamorphosis. The steroid hormone ecdysone has been shown to be crucial for these apoptotic events (Baehrecke 2000). During 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


early pupae
late pupae
Time (h) 0 1 2 3 4 5 6 7 8 12 13 15 16 17 18
2.2 kb ▶
rRNA ▶

B

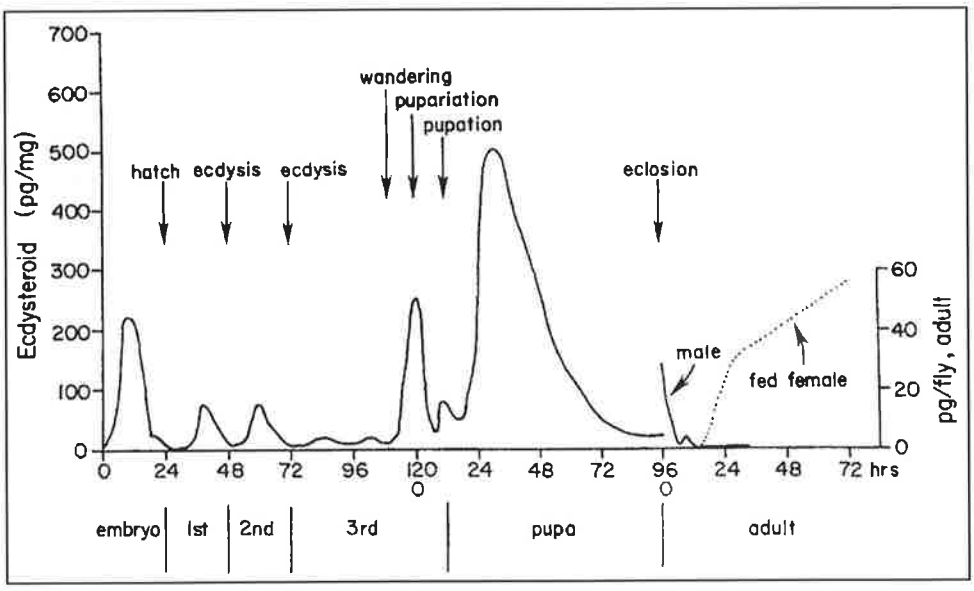
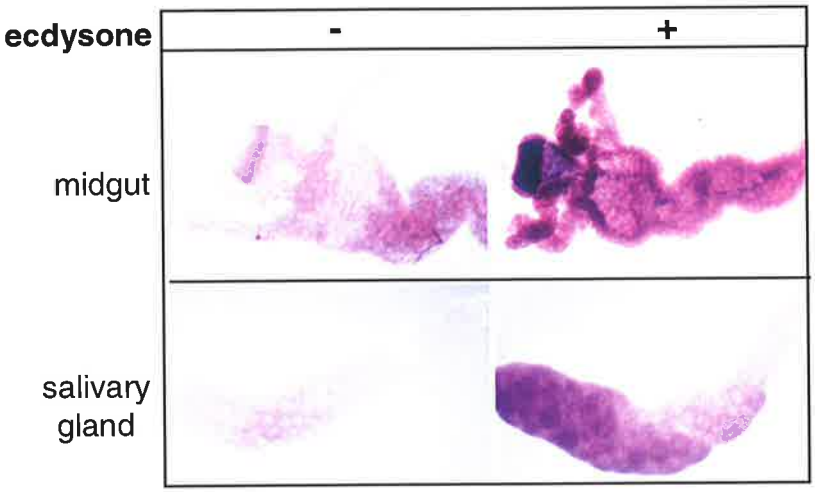

Ecdysteroid (pg/mg)
700
600
500
400
300
200
100
0
wandering
pupariation
pupation
hatch
ecdysis
ecdysis
eclosion
male
fed female
60
40
20
0
pg/fly, adult
0 24 48 72 96 120 24 48 72 96 24 48 72 hrs
0
0
embryo
1st
2nd
3rd
pupa
adult

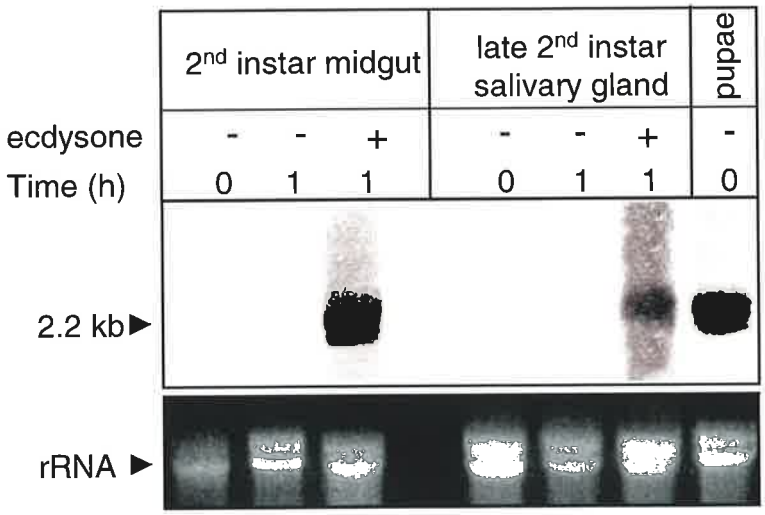
Figure 3.6. *dronc* mRNA is up-regulated by ecdysone.

(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


ecdysone
-
+
midgut
salivary gland

B


2nd instar midgut
late 2nd instar salivary gland
pupae
ecdysone
- - + - - + -
Time (h)
0 1 1 0 1 1 0
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 3.7A-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 3.8A). 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 $CuSO_4$, 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 $CuSO_4$ 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^{C318G} (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.

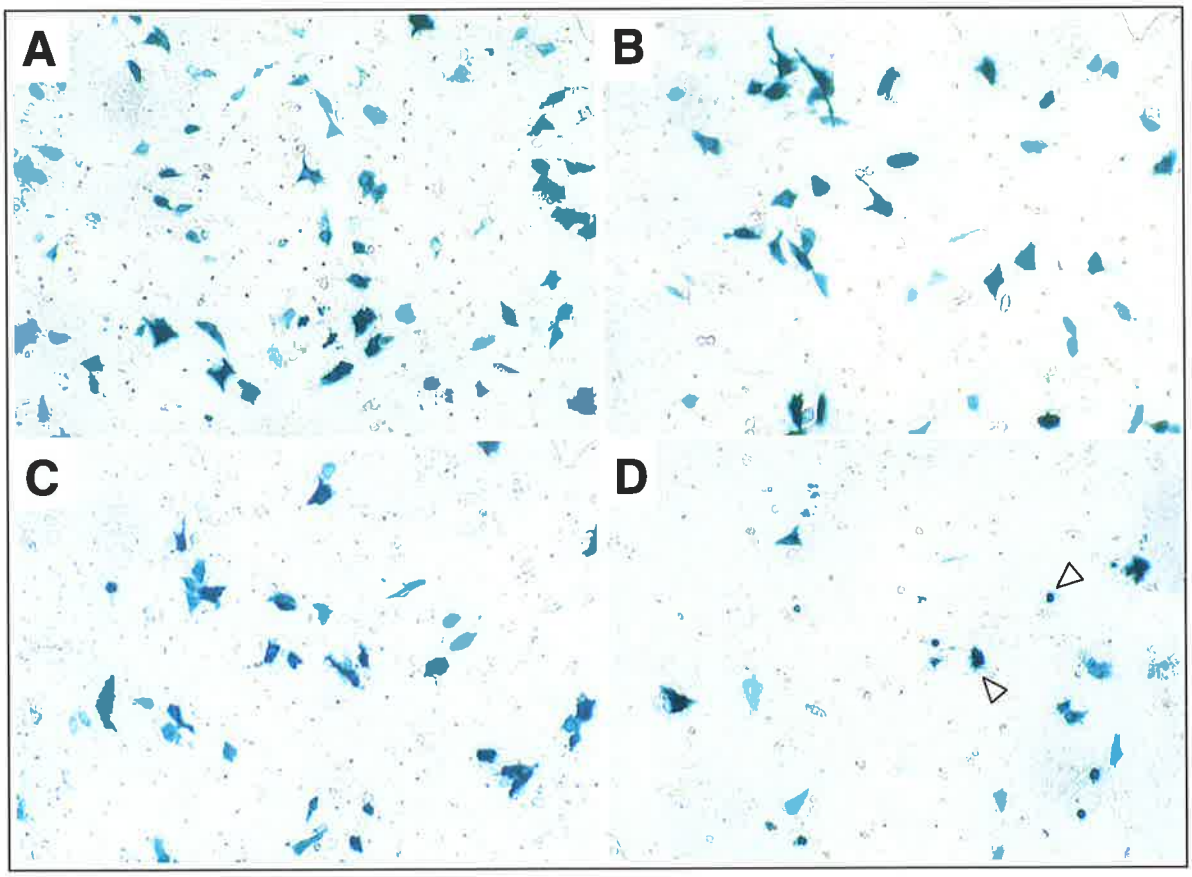
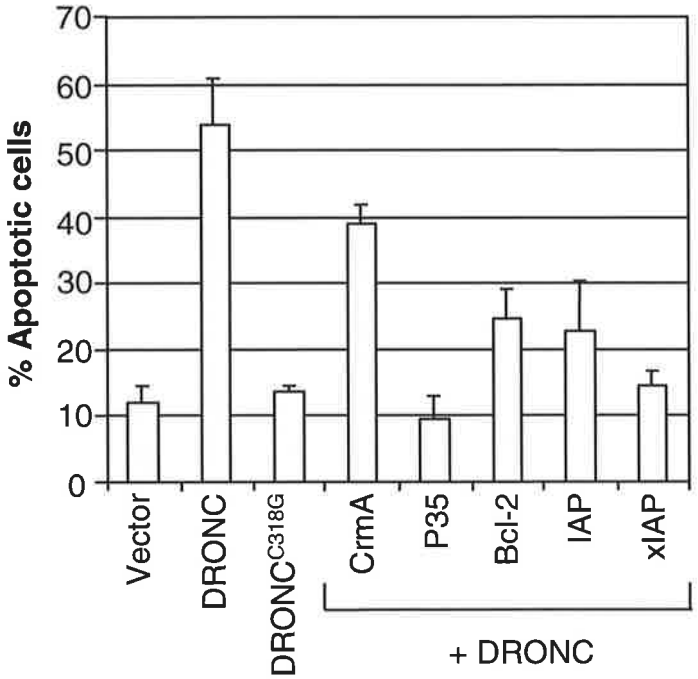

A
B
C
D

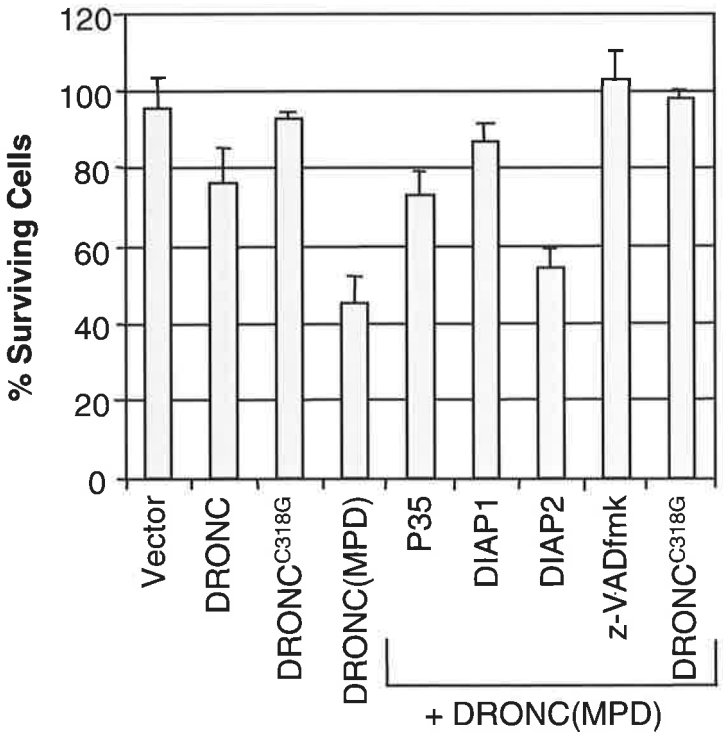
Figure 3.8 DRONC induces apoptosis in transfected cells.

(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 $CuSO_4$ (0.7 mM) for 48 h to induce DRONC expression. z-VAD-fmk (50μM) was added at the time of $CuSO_4$ addition. Cell survival was quantified by comparing the percentage of β-gal positive cells in $CuSO_4$ 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.

A


% Apoptotic cells
70
60
50
40
30
20
10
0
Vector
DRONC
DRONC^{C318G}
CrmA
P35
Bcl-2
IAP
xIAP
+ DRONC

B


% Surviving Cells
120
100
80
60
40
20
0
Vector
DRONC
DRONC^{C318G}
DRONC(MPD)
P35
DIAP1
DIAP2
z-VADfmk
DRONC^{C318G}
+ DRONC(MPD)

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 casapases. 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 $CuSO_4$ induction by immunostaining with α-DRONC antibody followed by α-rabbit-FITC conjugated antibody.

(A) pGFP vector at 24 h.

(B) pGFP-DRONC^{C318G} at 24 h.

(C) pGFP-DRONC at 24 h

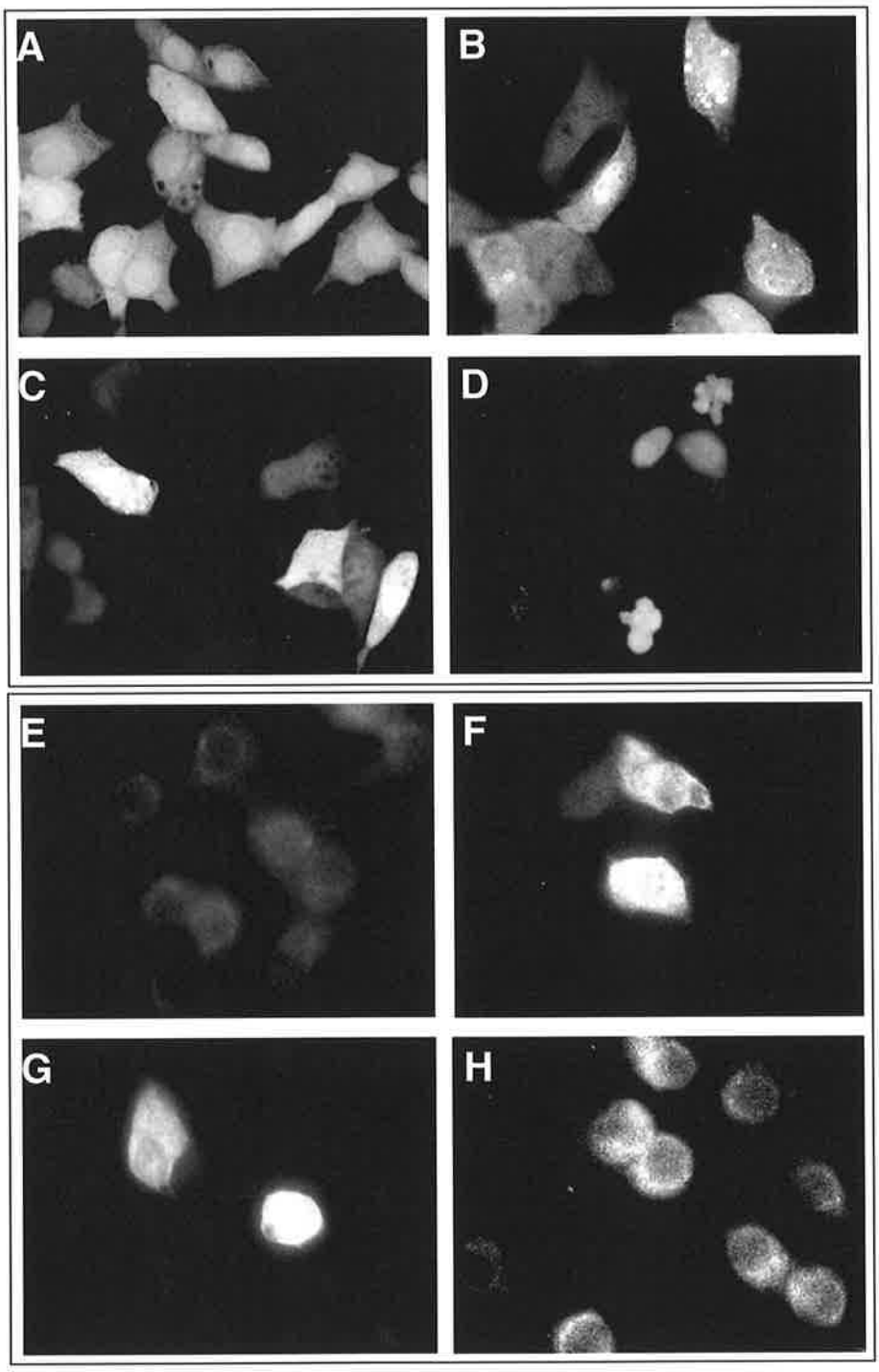
(D) pGFP-DRONC at 48h

(E) pRMHa3 vector

(F) pRMHa3-DRONC^{C318G}

(G) pRMHa3-DRONC

(H) pRMHa3-DRONC(MPD)


A
B
C
D
E
F
G
H

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 ^{35}S-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 DEXD 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.

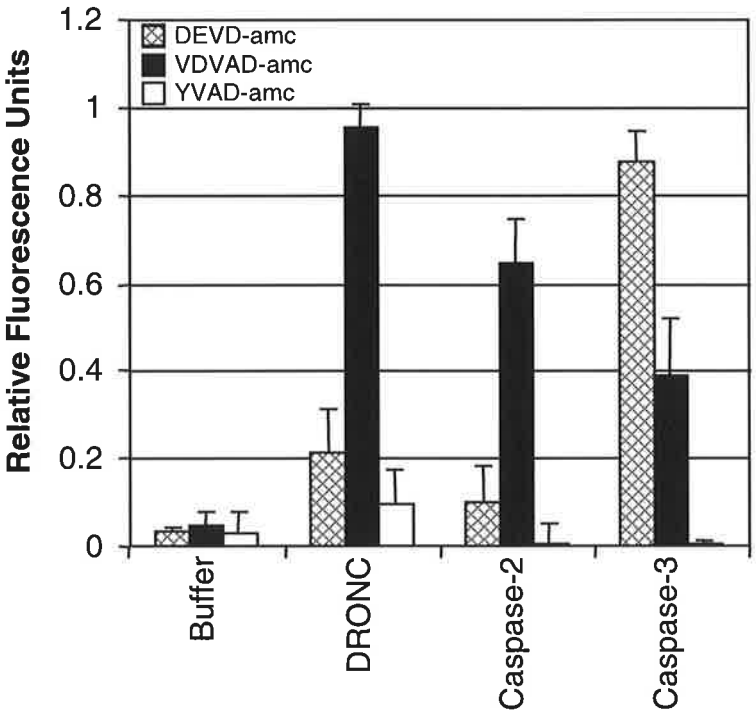
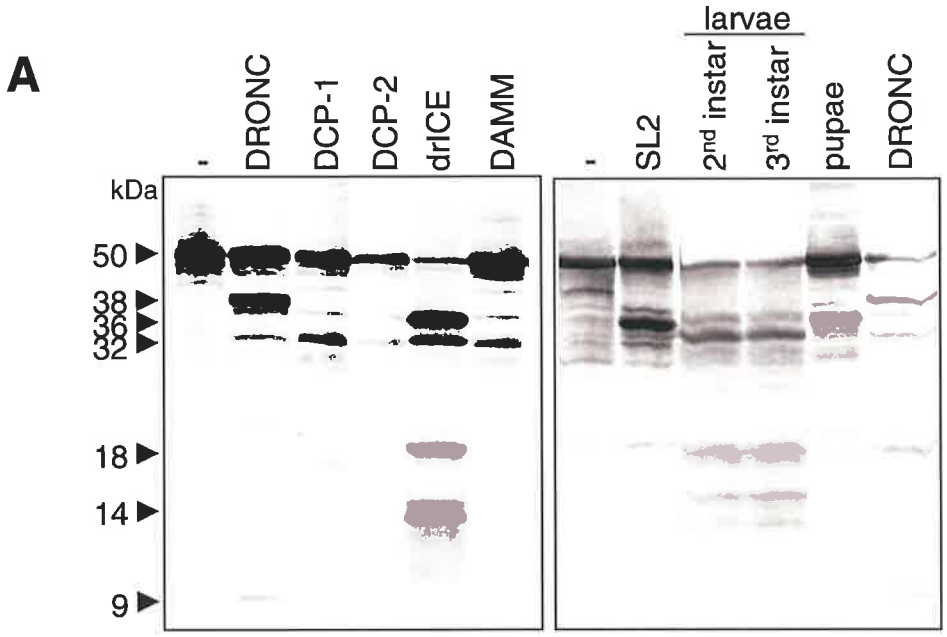

Relative Fluorescence Units
1.2
1
0.8
0.6
0.4
0.2
0
DEVD-amc
VDVAD-amc
YVAD-amc
Buffer
DRONC
Caspase-2
Caspase-3

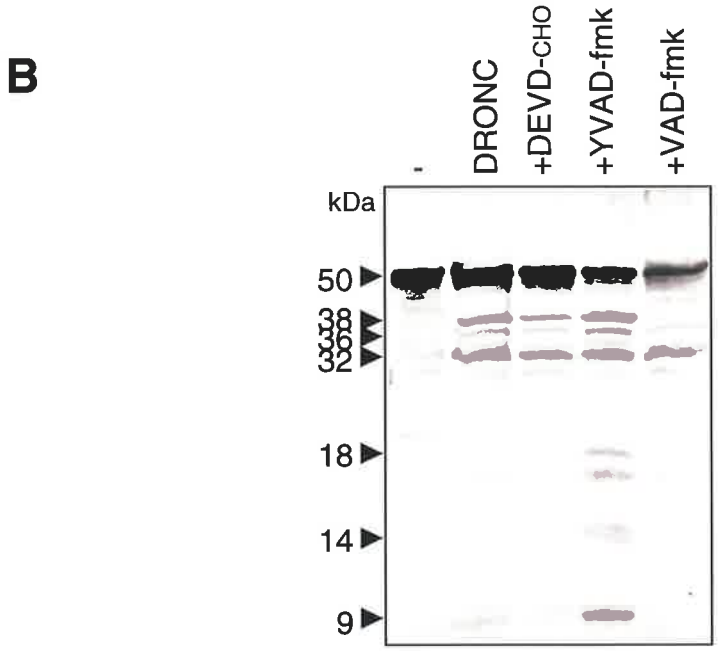
Figure 3.11 Processing of DRONC by active recombinant caspases and *Drosophila* lysates.

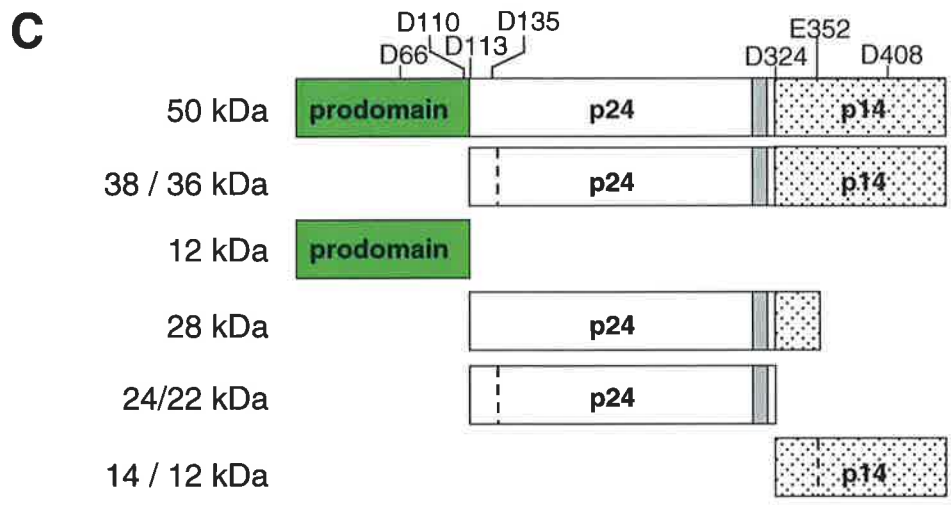
(A) ^{35}S-DRONC processing by *Drosophila* caspases, SL2 cell extract or larval extracts. *In vitro* translated, ^{35}S-labelled DRONC protein was incubated with the indicated caspases or lysates for 3 h at 37°C and processing analysed by autoradiography.

(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).


A
-
DRONC
DCP-1
DCP-2
drICE
DAMM
-
SL2
larvae
2nd instar
3rd instar
pupae
DRONC
kDa
50
38
36
32
18
14
9


B
-
DRONC
+DEVD-CHO
+YVAD-fmk
+VAD-fmk
kDa
50
38
36
32
18
14
9


C
D110
D135
D66
D113
E352
D324
D408
50 kDa
prodomain
p24
p14
38 / 36 kDa
p24
p14
12 kDa
prodomain
28 kDa
p24
24/22 kDa
p24
14 / 12 kDa
p14

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.

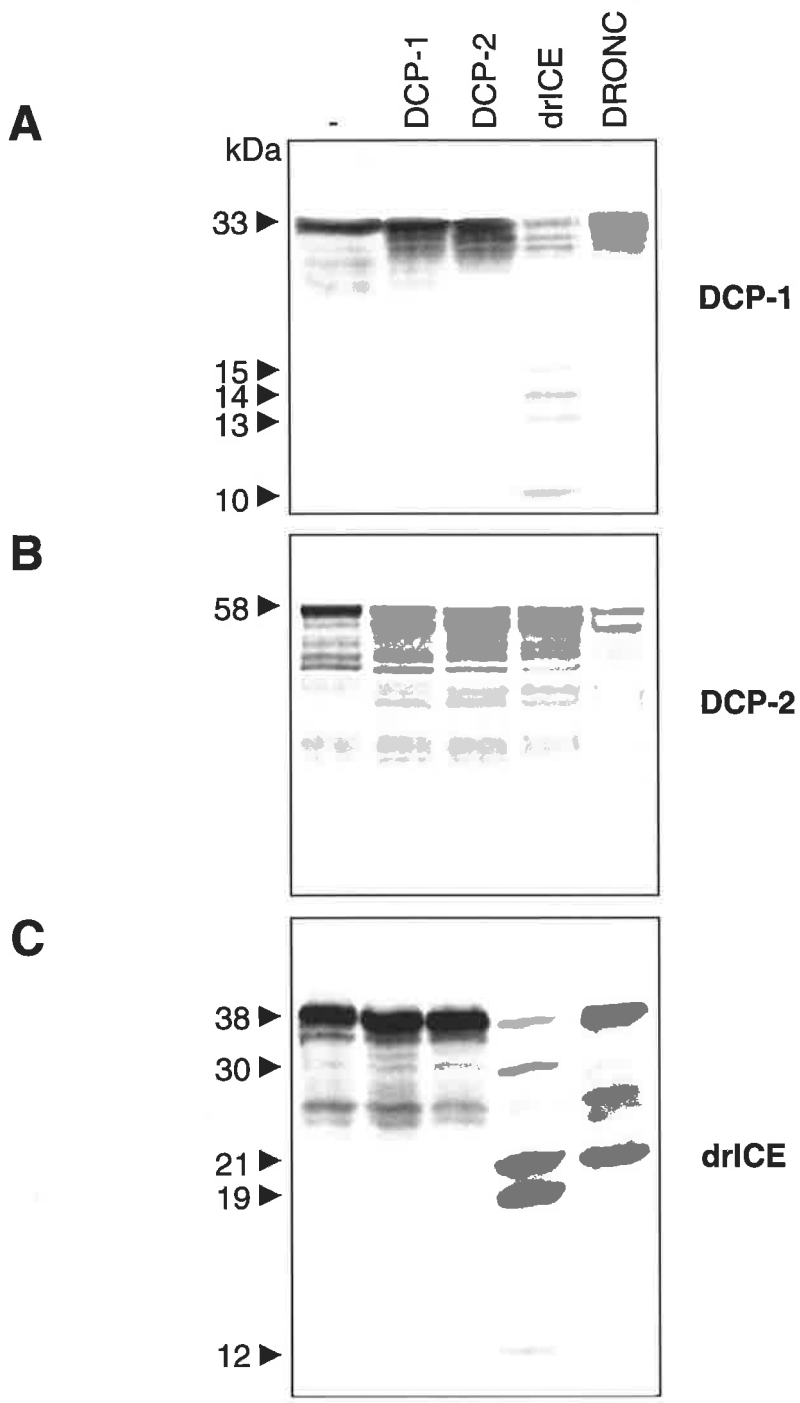
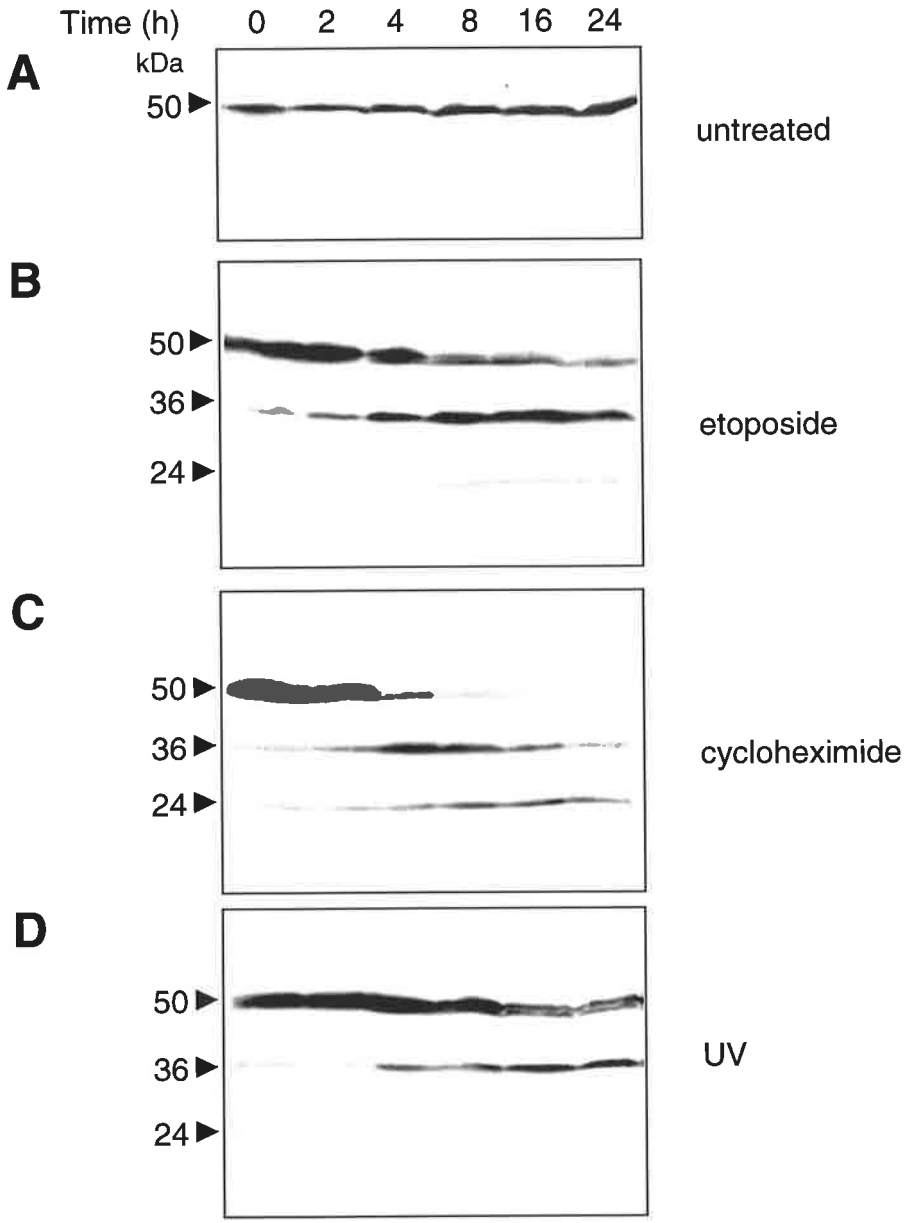

A
B
C
-
DCP-1
DCP-2
drICE
DRONC
kDa
33
15
14
13
10
58
38
30
21
19
12
DCP-1
DCP-2
drICE

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.


Time (h)
0
2
4
8
16
24
A
kDa
50
untreated
B
50
36
24
etoposide
C
50
36
24
cycloheximide
D
50
36
24
UV

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 cytochome *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 Baehecke 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 Baehecke 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 (Baehecke 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 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 aquired 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 Dm_0 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 colluegues (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 *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 *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 drICE.

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*^{C318G}, 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*^{C318G} 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*^{C318G} expression (Figure 4.1E). To analyse the effect of *dronc* expression during embryogenesis, *UAS-dronc* and *UAS-dronc*^{C318G} 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*^{C318G} 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*^{C318G} 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 *d*ronc induces cell death in transgenic flies.

UAS-dronc^{C318G}-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^{C318G}-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.

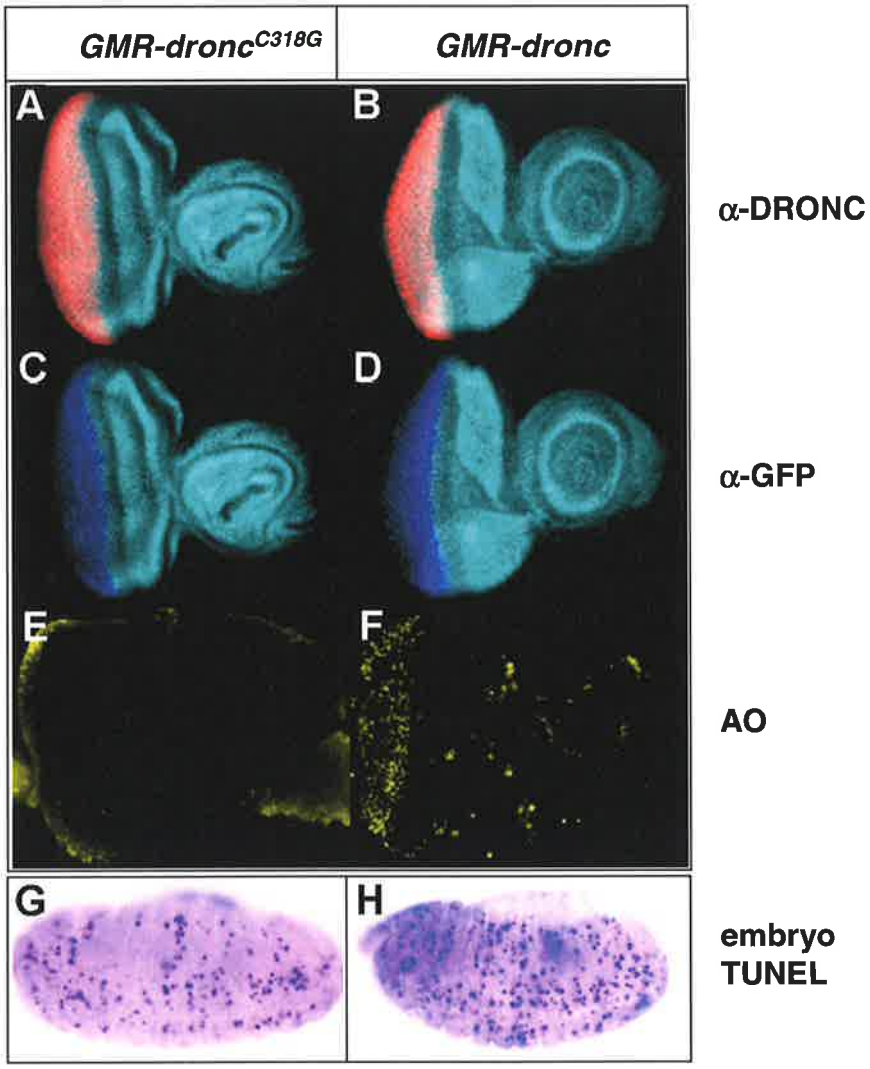
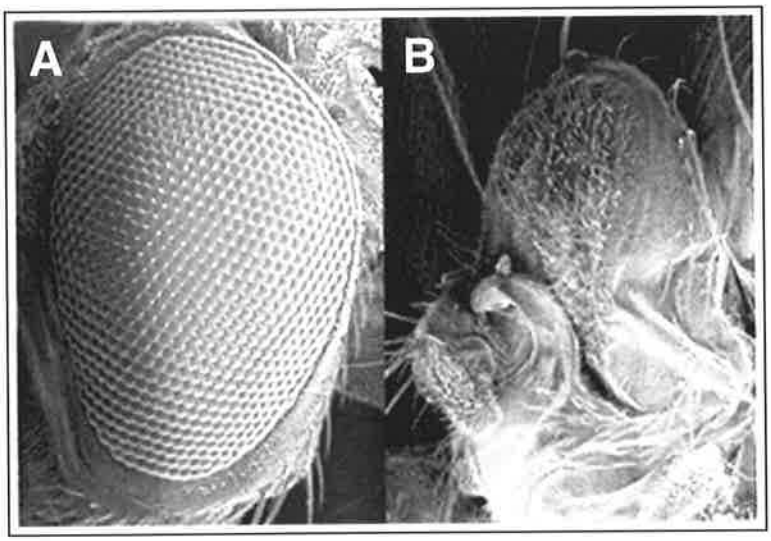

GMR-dronc^C318G
GMR-dronc
A
B
C
D
E
F
G
H
α-DRONC
α-GFP
AO
embryo
TUNEL

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^{C318G} ***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/His_6-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

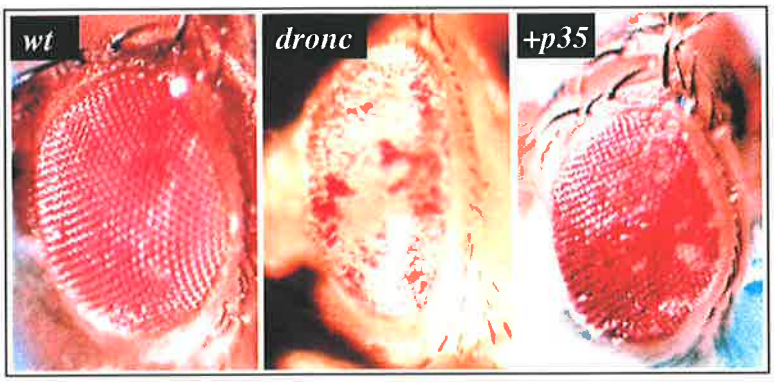
Figure 4.3 *p35* suppresses the *GMR-dronc* eye ablation phenotype.

(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 -His_6-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


wt
dronc
+p35

B

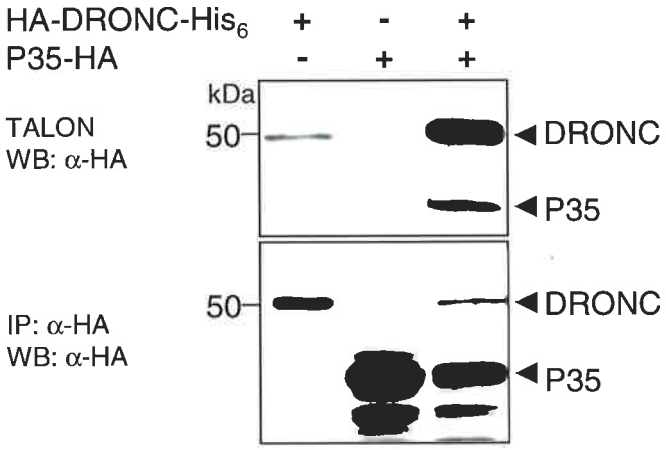

HA-DRONC-His6 + - +
P35-HA - + +
kDa
TALON
WB: α-HA
50
DRONC
P35
IP: α-HA
WB: α-HA
50
DRONC
P35

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/+* [$\boldsymbol{diap1^{-/-}}$]

- 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***].

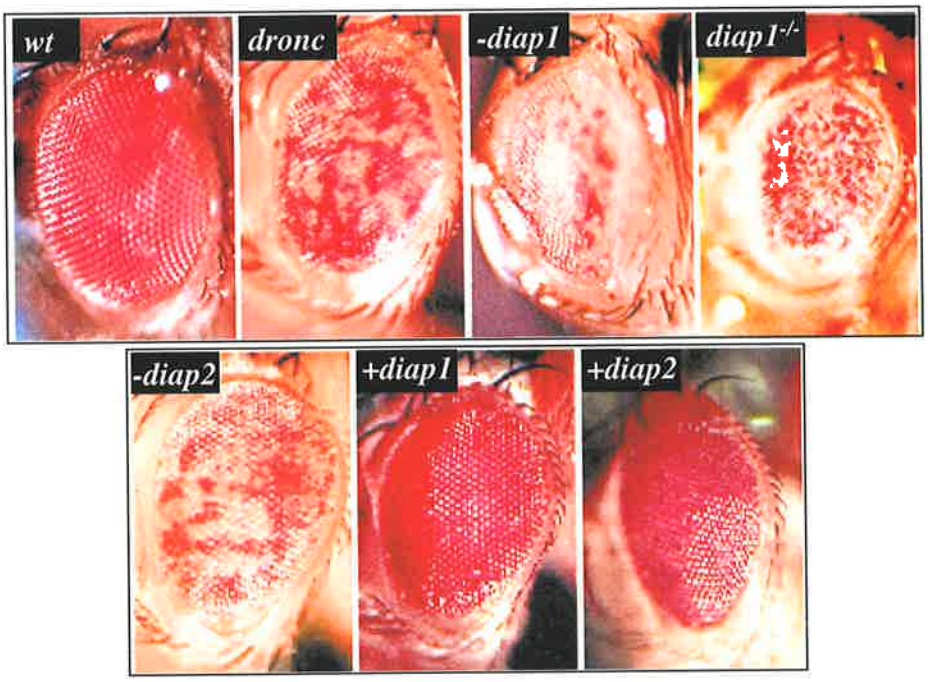
All crosses carried out at 25°C. Wild type (Canton-S) [***wt***] and *GMR-GAL4, UAS-dronc#80/+* [***dronc***] fly eyes are shown in comparison.

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

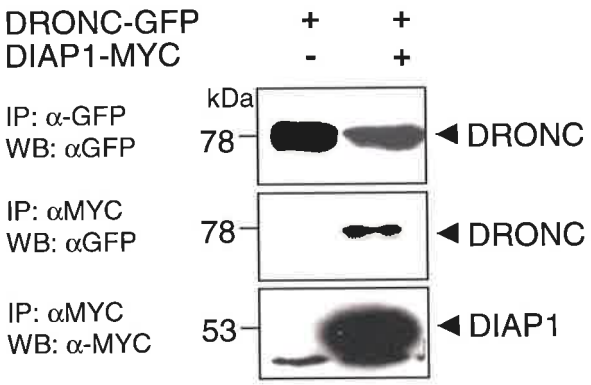
(B) Cell lysates were immunoprecipitated with α-MYC antibody (*middle and bottom panels*) and pelleted proteins were immunoblotted with α-GFP to detect DRONC (*middle panel*). (C) Cell lysates were immunoprecipitated with α-GFP antibody (*top* and *middle panels*) and protein complexes were immunoblotted 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_H), below DIAP1 and DIAP2 bands, is indicated.

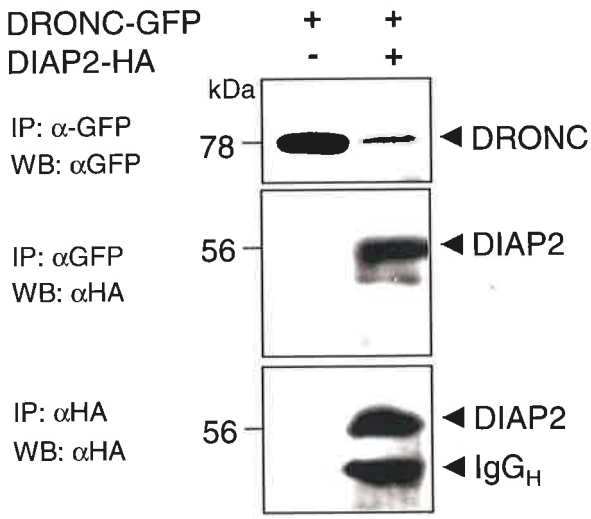
A


wt
dronc
-diap1
diap1-/-
-diap2
+diap1
+diap2

B


DRONC-GFP + +
DIAP1-MYC - +
kDa
IP: α-GFP
WB: αGFP
78
DRONC
IP: αMYC
WB: αGFP
78
DRONC
IP: αMYC
WB: α-MYC
53
DIAP1

C


DRONC-GFP + +
DIAP2-HA - +
kDa
IP: α-GFP
WB: αGFP
78
DRONC
IP: αGFP
WB: αHA
56
DIAP2
IP: αHA
WB: αHA
56
DIAP2
IgGH

(*thread*^{5}). 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.

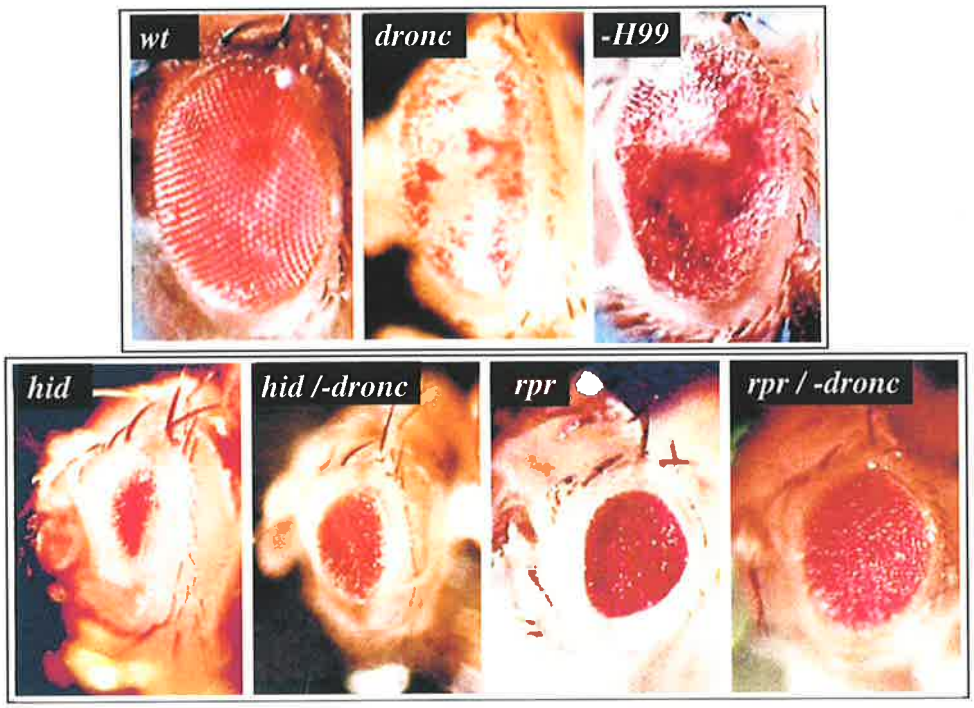
Expression of transfected RPR, HID or GRIM in *Drosophila* SL2 cells was very low, possibly due to their ability to efficiently induce apoptosis in these cells together with the poor transfection efficiency of SL2 cells. Due to the inability to pick up expression of

Figure 4.5 *dronc* genetically interacts with the *H99* genes and forms a protein complex with GRIM.

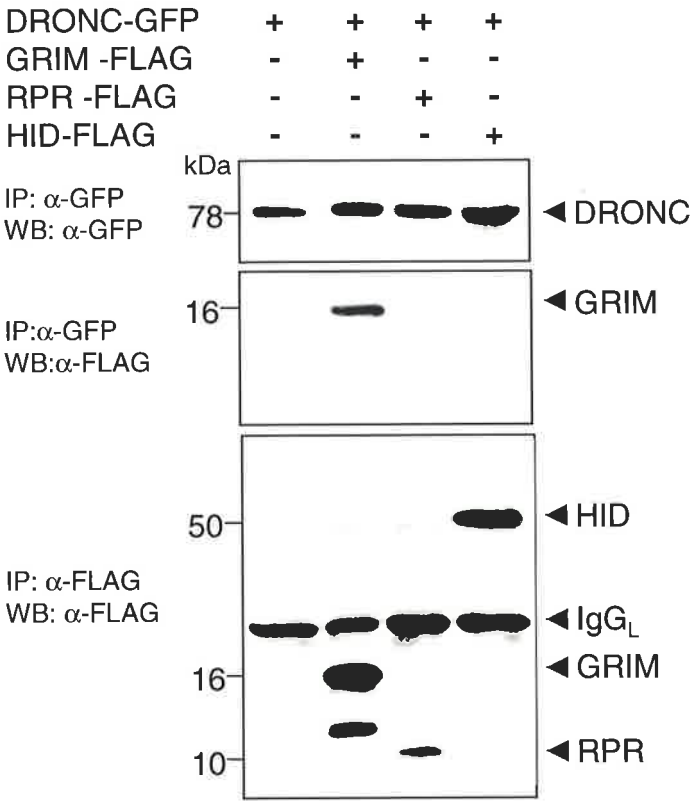
(A) *GMR-dronc#80/CyO* flies were crossed to flies containing a deficiency in the *H99* genes: *GMR-GAL4, UAS-dronc#80/+; Df(3L)H99/+* [***-H99***], at 29°C (*top panel*). *GMR-hid/+* [***hid***] or *GMR-rpr/+* [***rpr***] flies were crossed to flies containing a deficiency in the *dronc* gene: *GMR-hid/+; Df(3L)AC1/+* [***hid/-dronc***], *GMR-rpr/+; Df(3L)AC1/+* [***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_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


wt
dronc
-H99
hid
hid /-dronc
rpr
rpr / -dronc

B


DRONC-GFP + + + +
GRIM -FLAG - + - -
RPR -FLAG - - + -
HID-FLAG - - - +
kDa
IP: α-GFP
WB: α-GFP
78
DRONC
IP:α-GFP
WB:α-FLAG
16
GRIM
IP: α-FLAG
WB: α-FLAG
50
HID
IgG$_L$
16
GRIM
10
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^{C318G}-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.

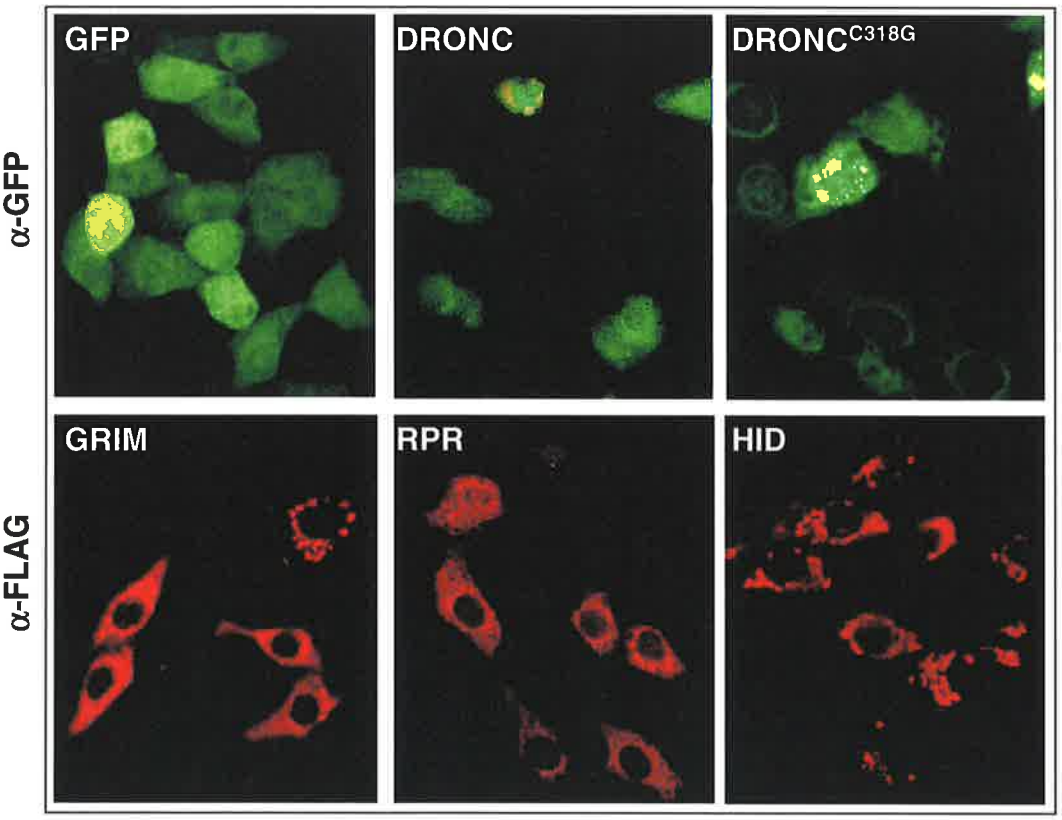
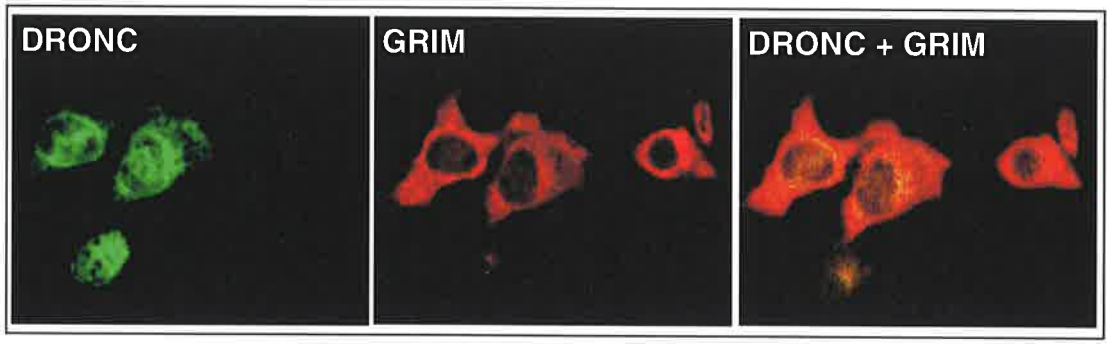

GFP
DRONC
DRONC^{C318G}
GRIM
RPR
HID
α-GFP
α-FLAG

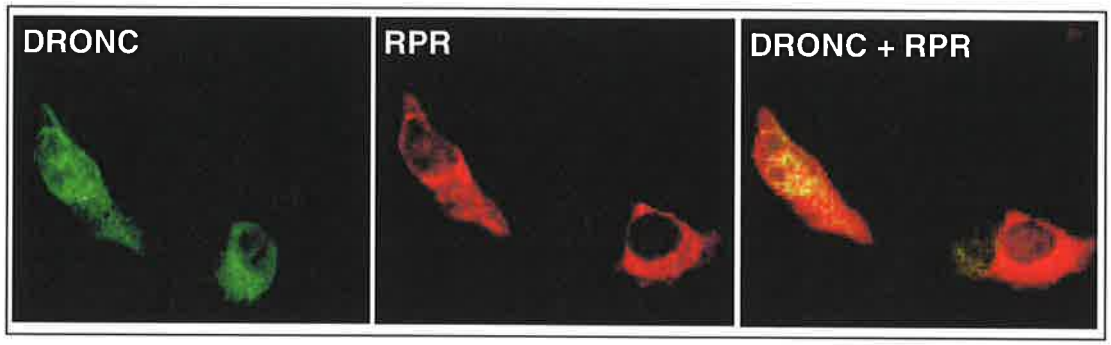
Figure 4.7 DRONC co-localises with HID in transfected 293T cells.

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*).

A


DRONC
GRIM
DRONC + GRIM

B


DRONC
RPR
DRONC + RPR

C

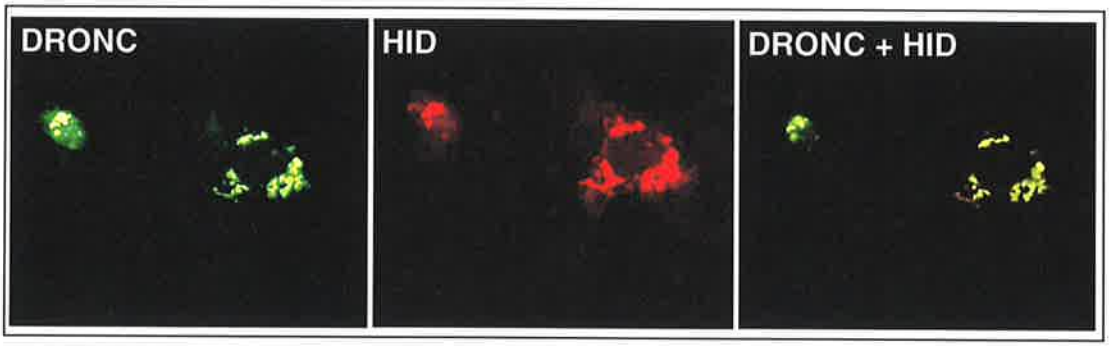
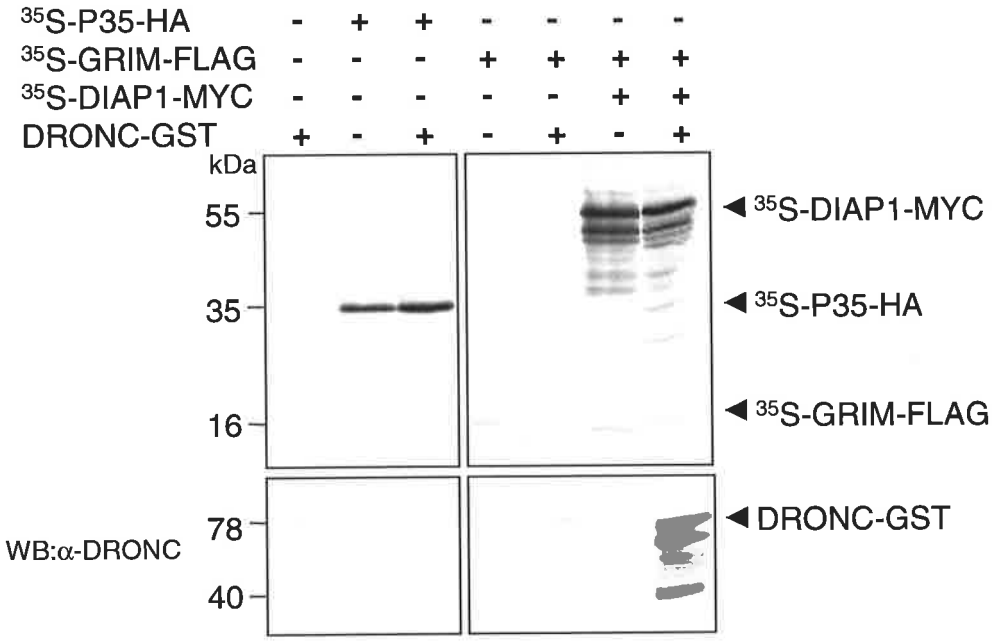

DRONC
HID
DRONC + HID

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*).


35S-P35-HA - + + - - - -
35S-GRIM-FLAG - - - + + + +
35S-DIAP1-MYC - - - - - + +
DRONC-GST + - + - + - +
kDa
55
35
16
78
40
WB:α-DRONC
35S-DIAP1-MYC
35S-P35-HA
35S-GRIM-FLAG
DRONC-GST

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 ^{35}S-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*^{CD4}, *dark*^{CD8} and *dark*$^{l(2)k11502}$ 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.

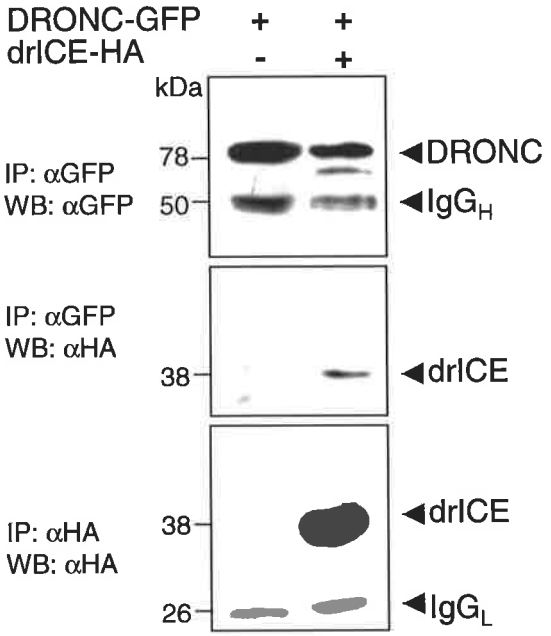
The next objective was to determine whether DRONC and DARK could physically interact with each other through immunoprecipitation reactions. Due to the low expression of the large 170 kDa full length protein in both SL2 and 293T cells, we used a truncation mutant of DARK, comprising amino acids 1-411, 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$^{1-411}$ 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 ^{35}S-labelled drICE-HA. ^{35}S-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 + +
drICE-HA - +
kDa
78
50
IP: αGFP
WB: αGFP
◀DRONC
◀IgG$_H$
IP: αGFP
WB: αHA
38
◀drICE
IP: αHA
WB: αHA
38
26
◀drICE
◀IgG$_L$

B

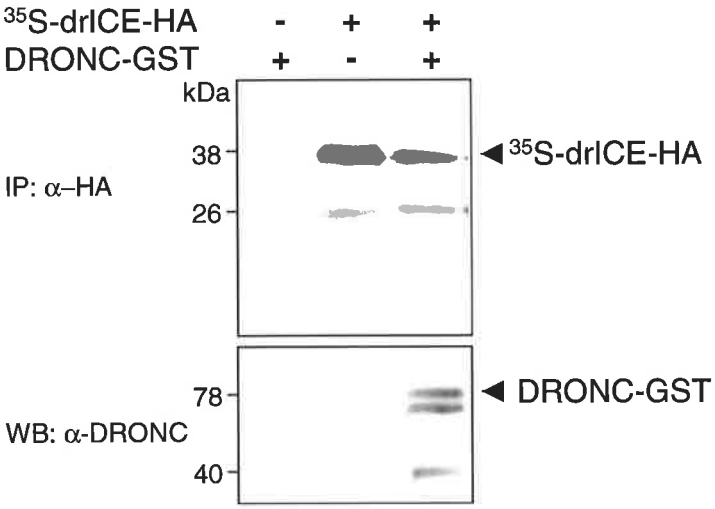

^{35}S-drICE-HA - + +
DRONC-GST + - +
kDa
38
26
IP: α–HA
◀^{35}S-drICE-HA
78
40
WB: α-DRONC
◀ DRONC-GST

Figure 4.10 DRONC interacts with DARK.

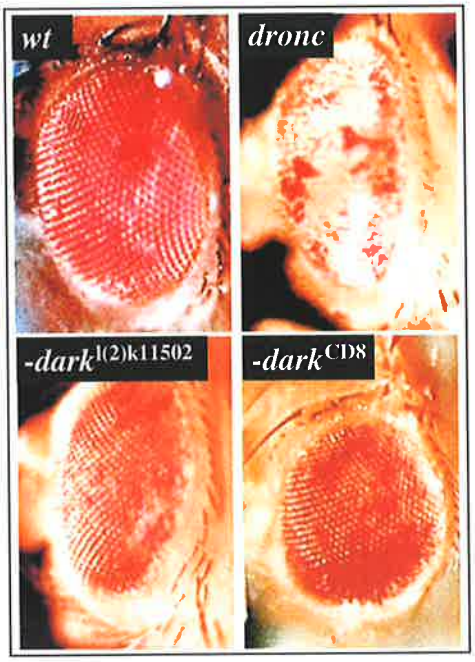
(A) *GMR-dronc#80/CyO* flies were crossed with *dark* P-element mutants [***-dark$^{l(2)k11502}$*** or ***-dark^{CD8}***] at 29°C, and the eye phenotype of the transheterozygous progeny analysed.

-(*top panel*): wild type (Canton-S) [***wt***] and *GMR-GAL4, UAS-dronc#80/+* [***dronc***]

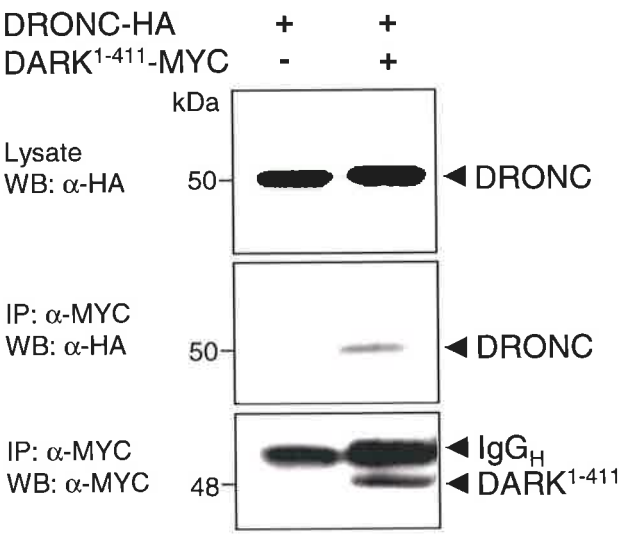
-(*bottom panel*): *GMR-GAL4, UAS-dronc#80/+; dark*$^{l(2)k11502}$*/+* [***dark$^{l(2)k11502}$***] and *GMR-GAL4, UAS-dronc#80/+; dark*^{CD8}*/+* [***dark^{CD8}***]

(B) SL2 cell lysates transfected with HA-tagged DRONC alone or with MYC-tagged DARK$^{1-411}$, 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. DARK$^{1-411}$ protein expression was detected by immunoprecipitating and immunoblotting with α-MYC antibody (*bottom panel*)

A


wt
dronc
-dark l(2)k11502
-dark CD8

B


DRONC-HA + +
DARK1-411-MYC - +
kDa
Lysate
WB: α-HA
50
DRONC
IP: α-MYC
WB: α-HA
50
DRONC
IP: α-MYC
WB: α-MYC
48
IgGH
DARK1-411

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

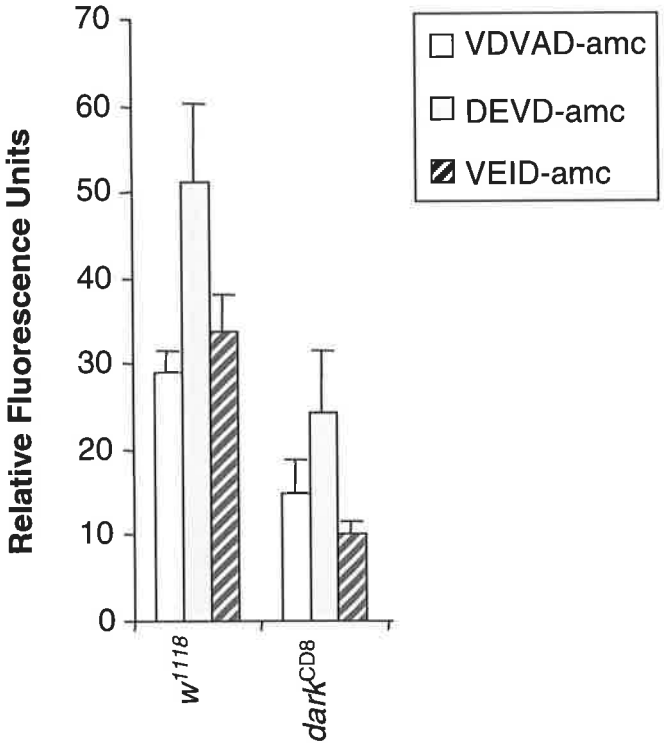
To test the requirement of DARK for DRONC activation, extracts from *dark*^{CD8} homozygous flies were prepared and assessed for their caspase activity and ability to cleave DRONC *in vitro*. As shown in Figure 4.11A, *dark* mutant flies had reduced caspase activity compared with wild type on DEVD-amc, 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 hypomorphic 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.

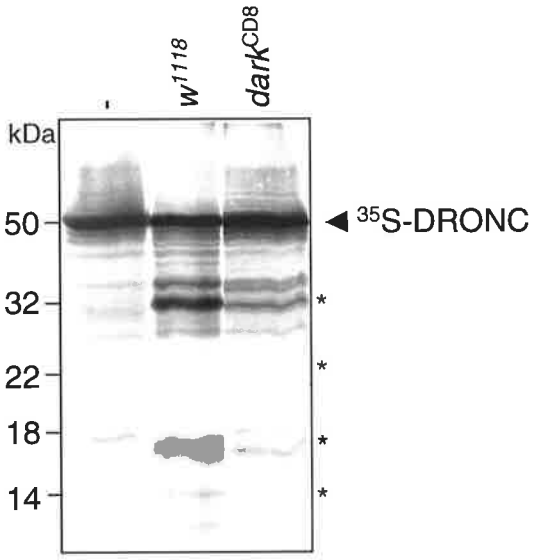
(A) *dark*^{CD8} has lower caspase activity. Caspase activity from *dark*^{CD8} mutant flies was compared with wild type (*w*^{1118}) flies by incubation with 100μM various fluorogenic caspase substrates, VDVAD-amc, DEVD-amc or VEID-amc at 37°C for 30 min. The release of -amc was monitored on a fluorimeter. An equivalent amount of protein was used in each sample. *dark*^{CD8} flies consistently had lower activity on the substrates assessed. Bars represent average fluorescence units ± SEM derived from three independent experiments.

(B) *dark*^{CD8} mutant extract has reduced ability to process DRONC. Protein lysates from *w*^{1118} or *dark*^{CD8} flies were incubated with *in vitro* translated ^{35}S-labelled DRONC for 3 h at 37°C and cleavage products were assessed by autoradiography. An equivalent amount of protein was used in each reaction. *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


Relative Fluorescence Units
70
60
50
40
30
20
10
0
VDVAD-amc
DEVD-amc
VEID-amc
w1118
darkCD8

B


-
w1118
darkCD8
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, ^{35}S-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.

I*n vitro* translated ^{35}S-labelled DRONC-HA was purified by immunoprecipitation with α-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.

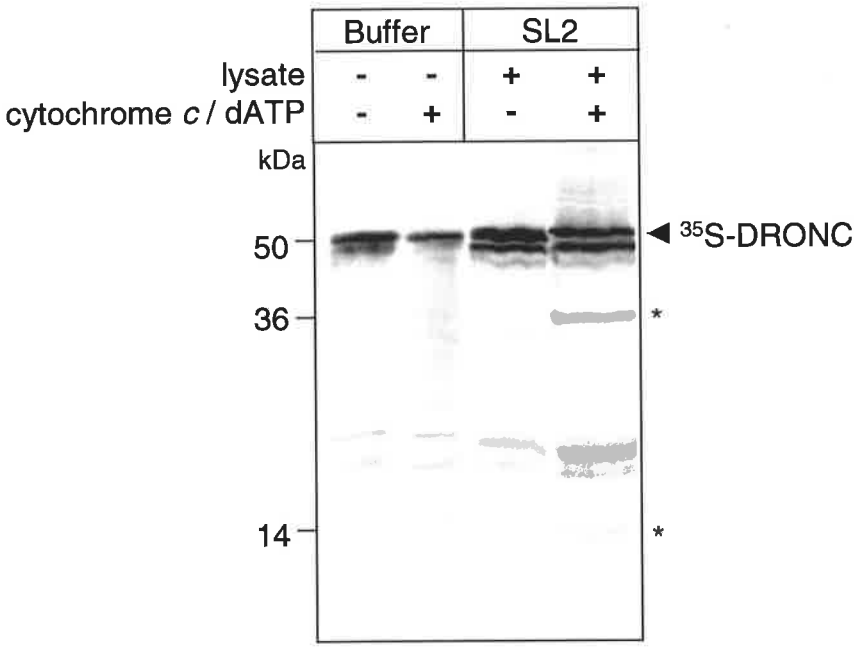
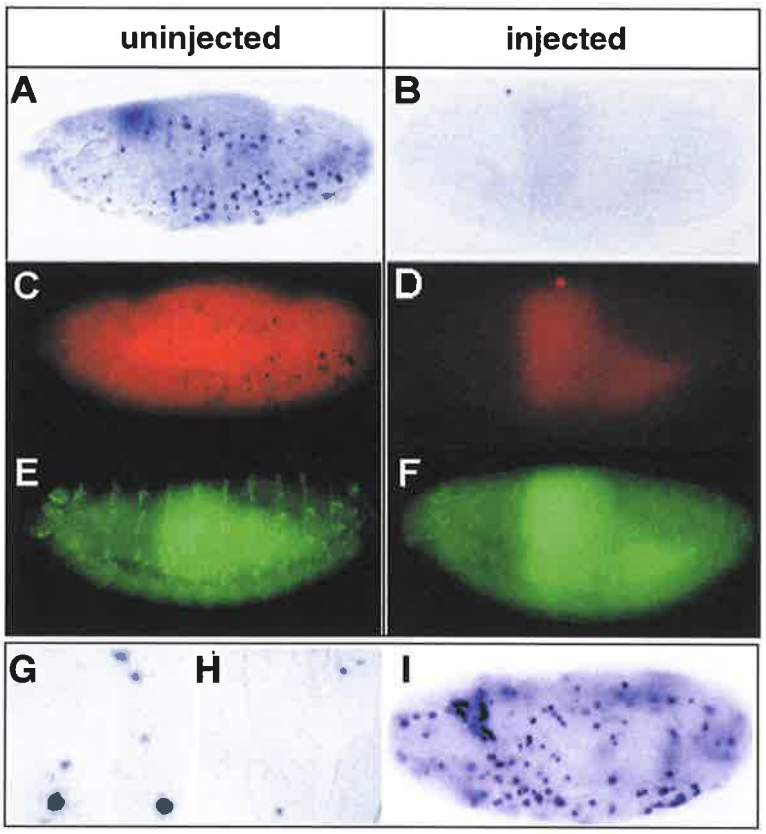

Buffer
SL2
lysate
cytochrome c / dATP
- - + +
- + - +
kDa
50
36
14
◀ 35S-DRONC
*
*

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.


uninjected
injected
A
B
C
D
E
F
G
H
I
TUNEL
α-DRONC
Mab 22C10
TUNEL

4.12 Discussion

This chapter demonstrates that DRONC mediates PCD *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 *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 *GMR-dronc* eye phenotype can be suppressed by *GMR-p35* and is sensitive to the dosage of the caspase inhibitor *diap1*, by the *H99* genes and by *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 *dark* mutant flies have reduced caspase activity and decreased ability to cleave DRONC and, that cytochrome *c* enhances DRONC processing to some extent, suggesting that DARK and cytochrome *c* are important for DRONC activation.

The suppression of *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 DIAP1, 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-x_L 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.

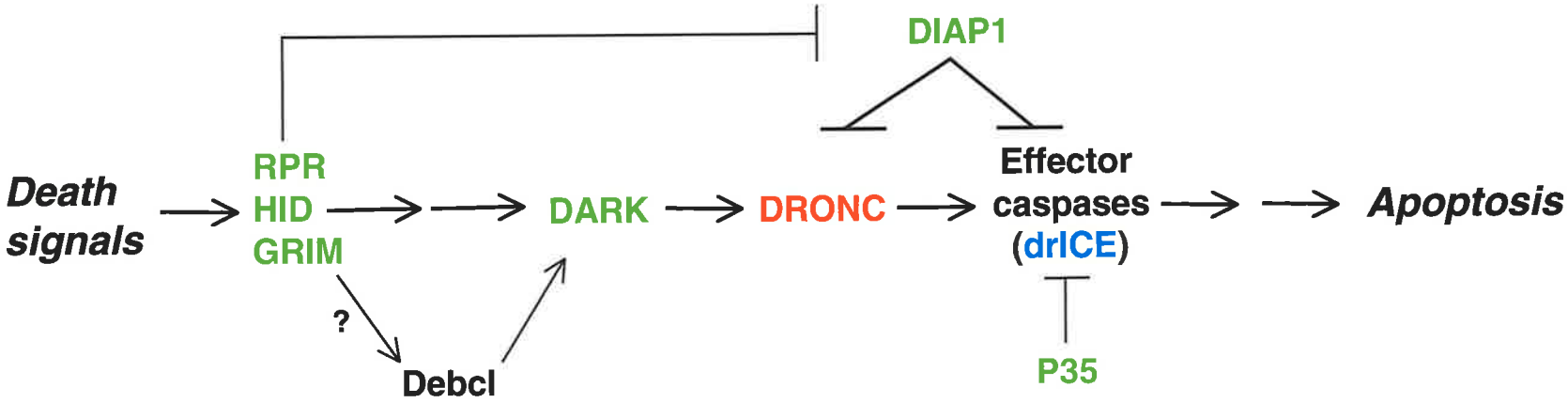
The *Drosophila* inhibitor of apoptosis DIAP1 inhibits the activity of drICE and DCP-1 and is antagonised by RPR, HID and GRIM (Hawkins *et al.* 1999; Wang *et al.* 1999). Our data show a dosage-dependent enhancement of *GMR-dronc* by a *diap1* mutation and additionally show that DRONC and DIAP1 form 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 *diap1* 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 *diap1* 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.


DIAP1
Death signals
RPR
HID
GRIM
DARK
DRONC
Effector caspases (drICE)
Apoptosis
?
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 Dm_0 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 thee exons (Figure 5.1A). The complete mRNA coding sequence is displayed in Figure 5.1B. The chomosomal location of DECAY was determined by hybridisation to a filter comprising *Drosophila* genomic P1 clones. The *decay* gene was localised to the Fas1 contig on chomosome 3, region 89C6-D4 (data not shown). None of the other published *Drosophila* caspase genes map to or near this region.

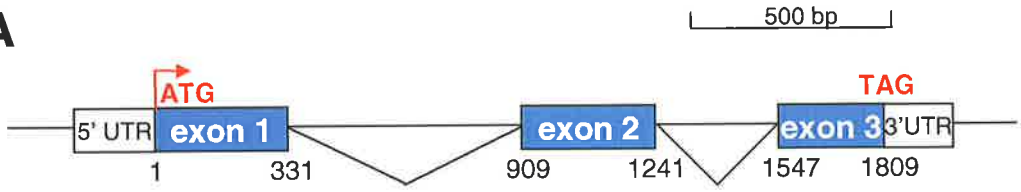
DECAY encodes a 308 amino acid protein (Figure 5.2A-C). *In vitro* translation of DECAY produced a 35 kDa protein consistent with its expected size (Figure 5.2B). DECAY shares greatest homology, 39% identity (54% similarity), with *Spodoptera frugiperda* Caspase-1. Of the mammalian caspases, DECAY shares 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



B

```
   1 ggacggtcag aaatggacga caccgacttc tcgctcttcg ggcagaagaa caagcacaag
  61 aaggacaagg cggatgccac caagatcgcc catacgccca catcggagct ggacctcaaa
 121 aggatcataa tctcgcgccc caccaacgag gacacatacg agaattgcgc gcgagcgggc
 181 attgcgctaa tcctgaacca caaggatgtc aagggacaga agcagcgcgt gggcaccgaa
 241 cgggatcgcg atgacatgga ggcgacgctg cagggattcg gattcgatgt acgcaccttc
 301 gacgatctga ccttctccga gatcaacgac acgctcaaag aggttgctcg ggaggatcac
 361 agtcaaaacg actgctttgt gttggcggtg atgtcacacg gcaccgaagg caaggtctac
 421 gctaaggaca tgtcatatcc tgtggagcgc ctttggaatc ccttcctcgg cgacaactgc
 481 aagacgctca agaacaaacc caaactcttc ttcatccagg cctgtcgcgg agccaacctg
 541 gaaaaggcag tcgagttctc cagcttcgct gtgatgacca gggaactggt cccggagccc
 601 gctgccgccg ttcagcccat cacctatgcc attcccagca cggcggacat cctcgtcttc
 661 tactccacat tcgacaaatt cttctcattc cgtaacgttg acgatggctc ctggttcatc
 721 cagagcctgt gccgcgtcct cgaccaggcc gccgccaacg aagctgccac gccggagggc
 781 gtcgagctac tccgcctgct gaccgcagtg aatcggaagg tggcctacga gtaccagtcg
 841 aatacgaaga acgaggccct caaccagatg aaggaaatgc ccaactttat gtcgacactg
 901 accaaaacat tccagctgcg tgttaagccc aagaccgtga ctccggtcac gaaatcagcg
 961 acaatccaat cgggactgtg agtcagtccg cgggtcaaga acgaaggacg ccacaactct
1021 ttcatttaat cgtaatgttt gcaatttgtt aatagaaata catatcgatg gcacttaaaa
1081 aaaaaaaaaa aaaaaa
```

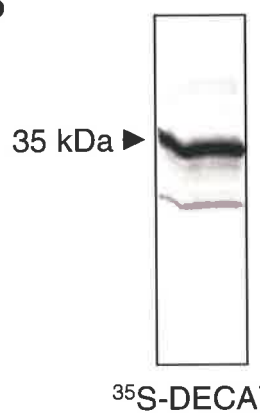
Figure 5.2 DECAY sequence and its homology to other *Drosophila* caspases.

(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.

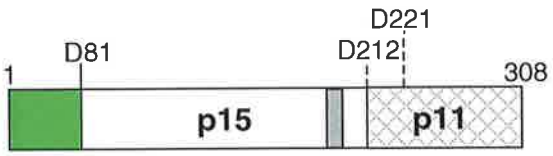
A

```
MDDTDFSLFG QKNKHKKDKA DATKIAHTPT SELDLKRIII SRPTNEDTYE NCARAGIALI  60
LNHKDVKGQK QRVGTERDRD DMEATLQGFG FDVRTFDDLT FSEINDTLKE VAREDHSQND 120
CFVLAVMSHG TEGKVYAKDM SYPVERLWNP FLGDNCKTLK NKPKLFFIQA CRGANLEKAV 180
EFSSFAVMTR ELVPEPAAAV QPITYAIPST ADILVFYSTF DKFFSFRNVD DGSWFIQSLC 240
RVLDQAAANE AATPEGVELL RLLTAVNRKV AYEYQSNTKN EALNQMKEMP NFMSTLTKTF 300
QLRVKPKT                                                          308
```

B



C



D

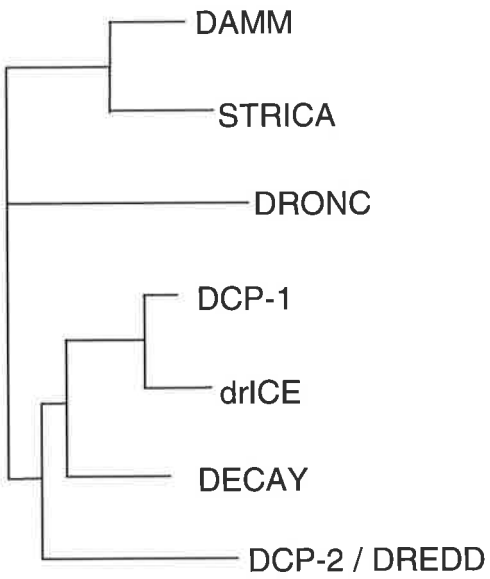
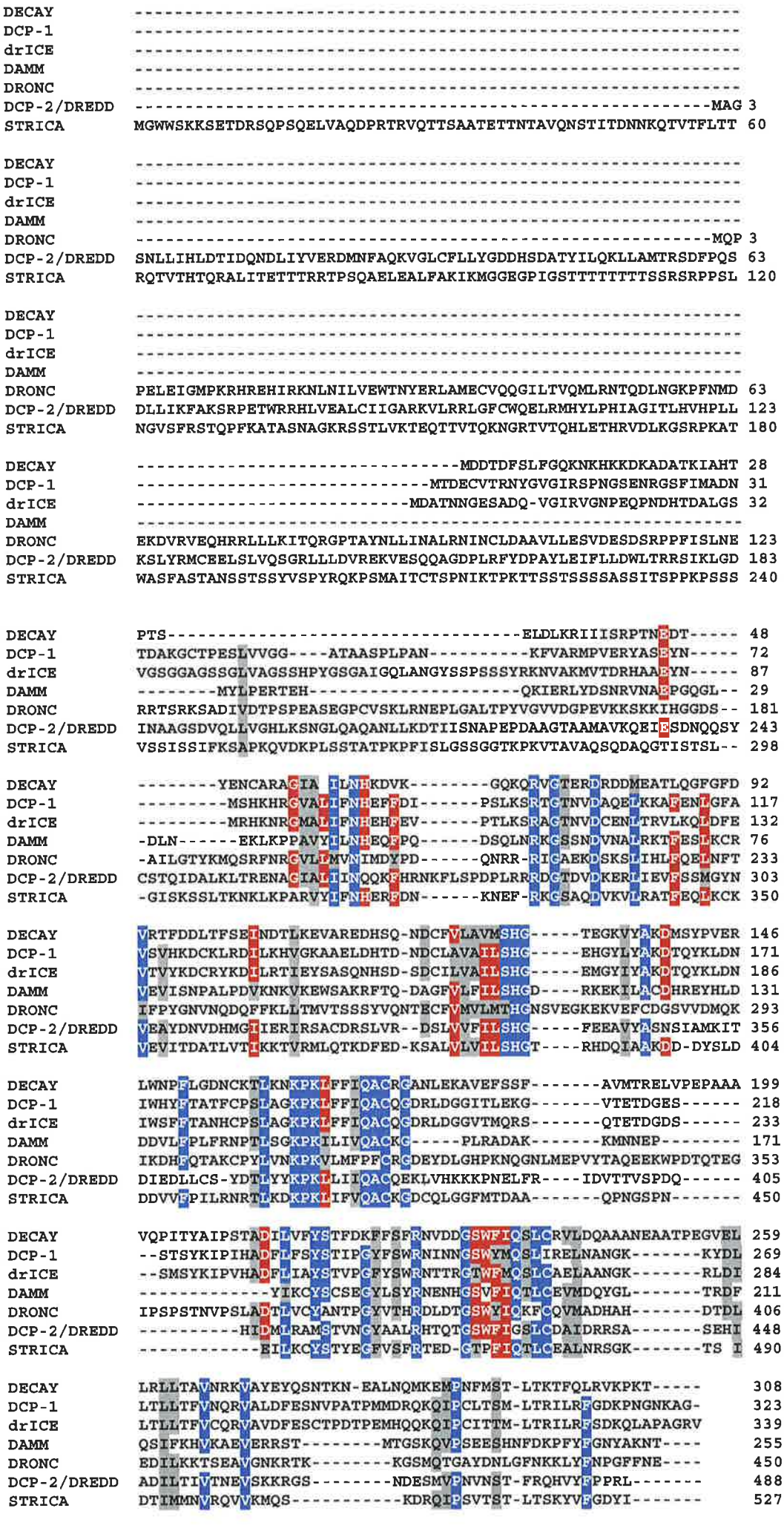


Figure 5.3 Alignment of *Drosophila* caspases.

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.


DECAY
DCP-1
drICE
DAMM
DRONC
DCP-2/DREDD
STRICA

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

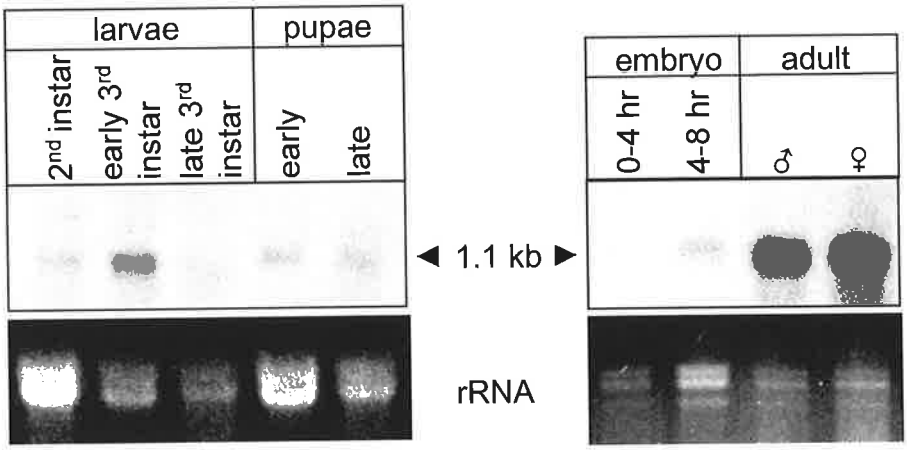
RNA blot analysis detected a *decay* transcript of approximately 1.1 kilobase in size in most developmental stages; larvae, pupae and adult fly (Figure 5.4A). Highest expression of *decay* was detected during early third instar larval stage when developmental apoptosis is occurring. Due to low levels 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.

A


larvae
pupae
2nd instar
early 3rd instar
late 3rd instar
early
late
embryo
adult
0-4 hr
4-8 hr
♂
♀
◄ 1.1 kb ►
rRNA

B

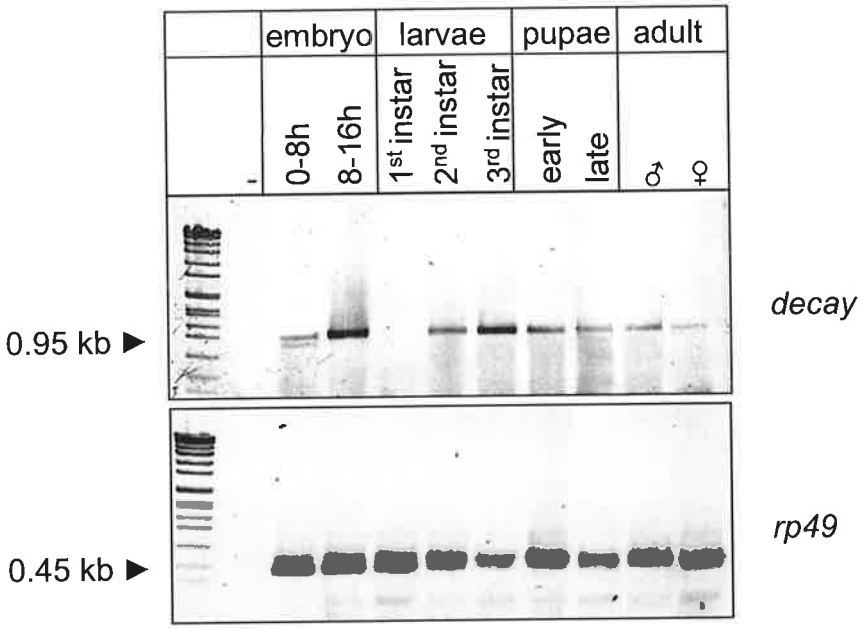

embryo
larvae
pupae
adult
0-8h
8-16h
1st instar
2nd instar
3rd instar
early
late
♂
♀
0.95 kb ►
decay
0.45 kb ►
rp49

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.

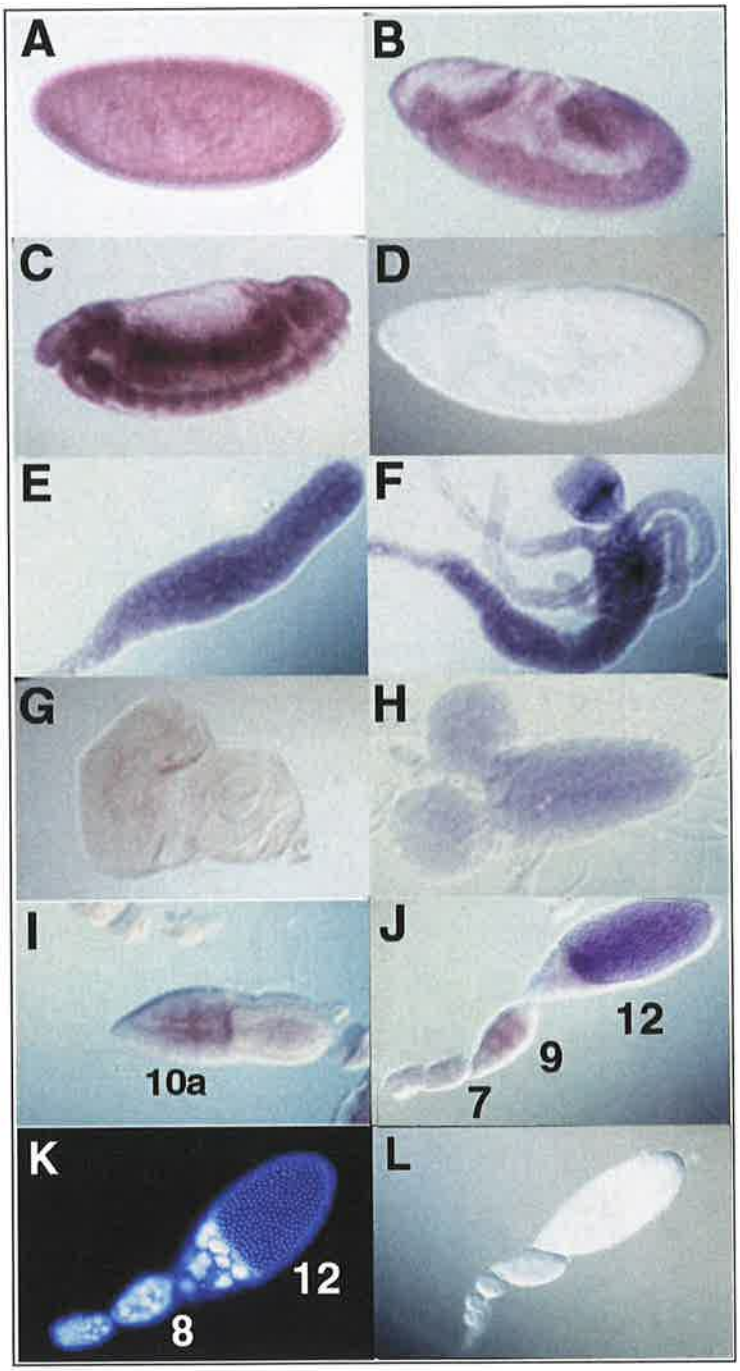
decay sense control on third instar larval tissues showed no staining (data not shown).

(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.


A
B
C
D
E
F
G
H
I
10a
J
12
9
7
K
12
8
L

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

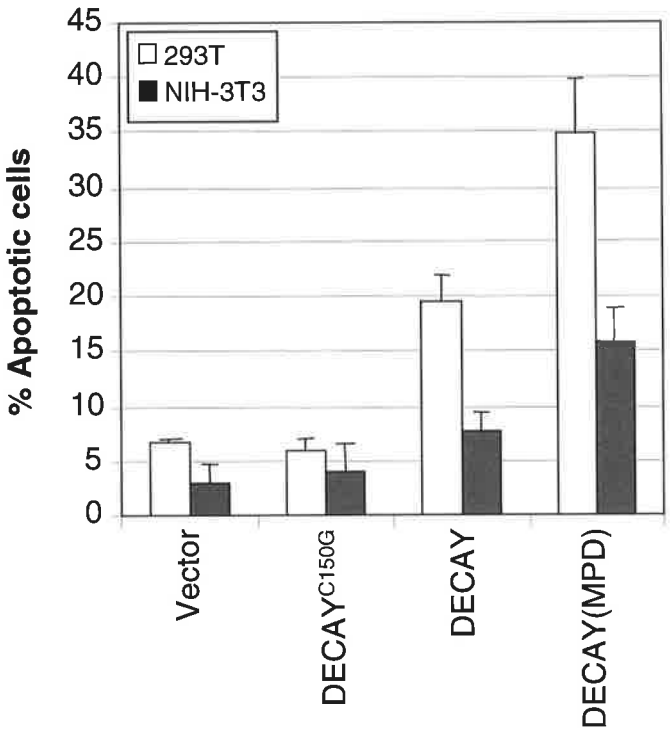
Many caspases, when overexpressed in cultured cells, induce apoptosis to some degree. We therefore analysed whether DECAY was able to induce apoptosis in transfected cells. In 293T cells, at either 24 or 48 h following transfection, full length DECAY was unable to induce a significant level of apoptosis. 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 cyteine mutant ($DECAY^{C150G}$) 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.

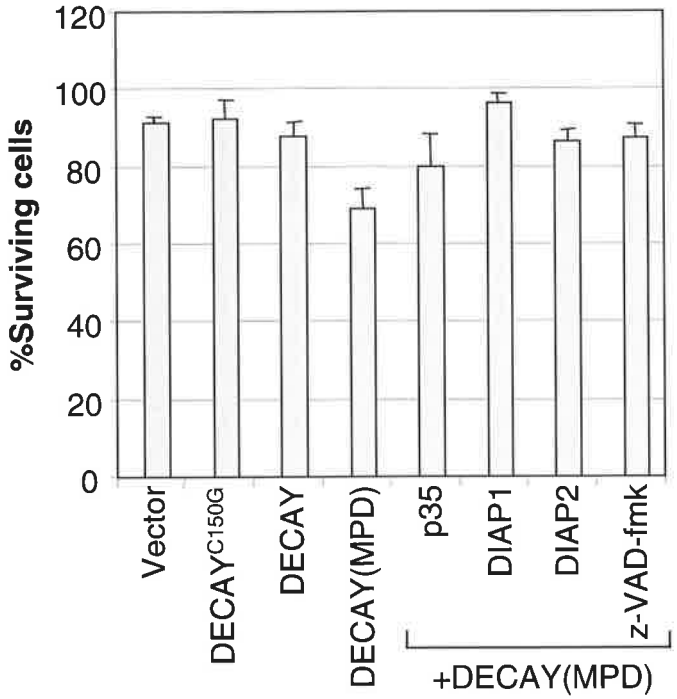
(A) Various expression constructs were co-transfected with pEF-β-gal into 293T or NIH-3T3 cells and 24 h post-transfection, cells were fixed and stained with X-gal and were observed for apoptosis. (B) *Drosophila* SL2 cells were co-transfected with various pRMHa3 vector expression constructs as labelled and pCasper-lacZ. 24 h after transfection cells were heat shocked to induce lacZ expression and then treated with $CuSO_4$ (0.7mM) for 48 h to induce expression of transfected constructs. Cell survival was quantified by comparing the percentage β-gal positive cells in $CuSO_4$ 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


293T
NIH-3T3
% Apoptotic cells
45
40
35
30
25
20
15
10
5
0
Vector
DECAY^{C150G}
DECAY
DECAY(MPD)

B


%Surviving cells
120
100
80
60
40
20
0
Vector
DECAY^{C150G}
DECAY
DECAY(MPD)
p35
DIAP1
DIAP2
z-VAD-fmk
+DECAY(MPD)

unable to induce a significant level of death 48 h after $CuSO_4$ 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); $DECAY^{C150G}$-FLAG (F); DECAY-FLAG (G) or DECAY(MPD)-FLAG (H). 24 h after transfection cells were $CuSO_4$ induced and left for a further 24 h before fixation and antibody staining as described above.

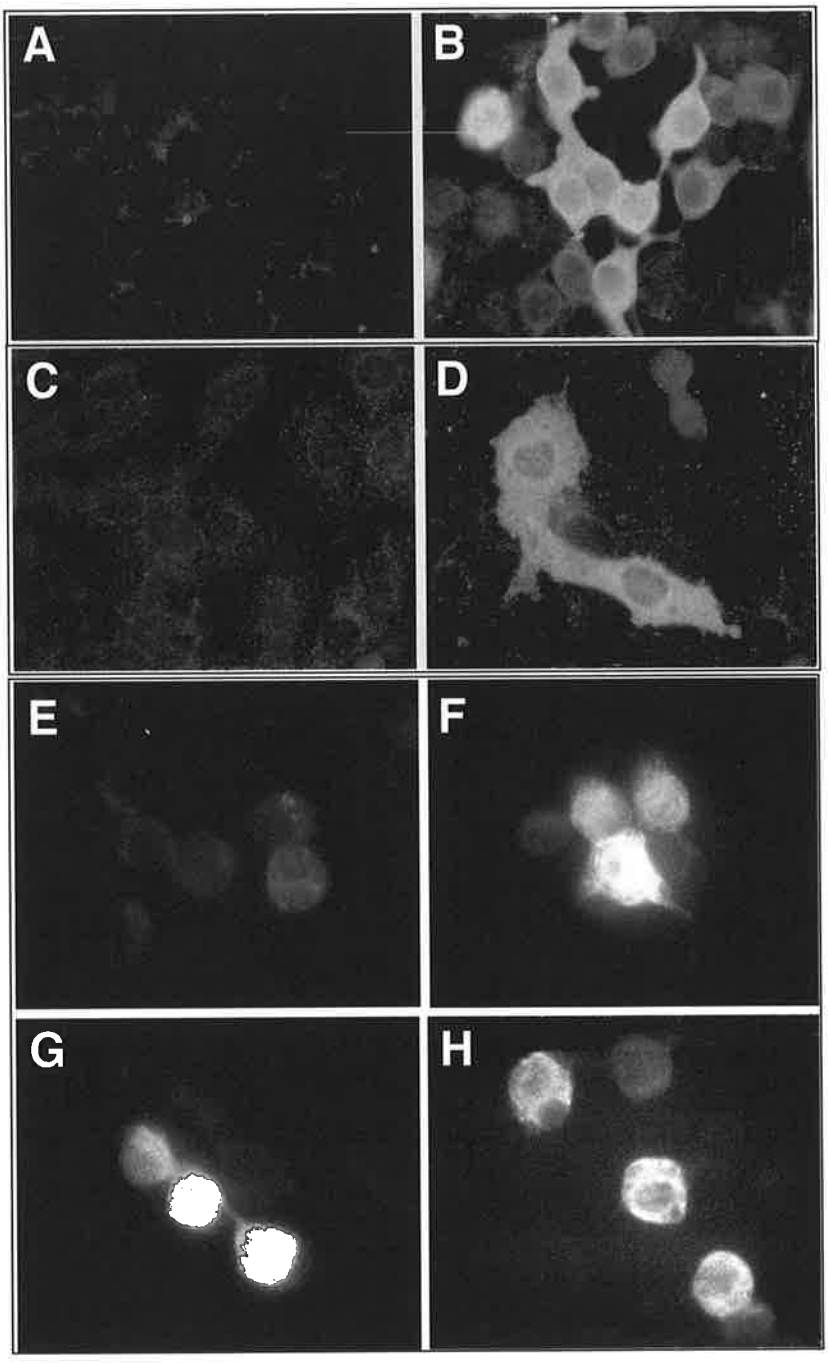
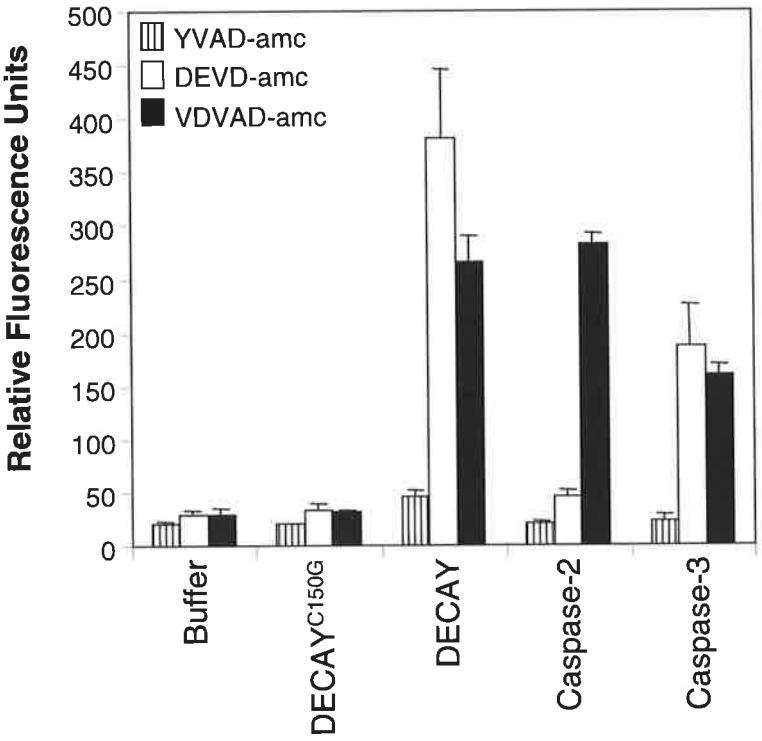

A
B
293T
C
D
NIH-3T3
E
F
SL2
G
H

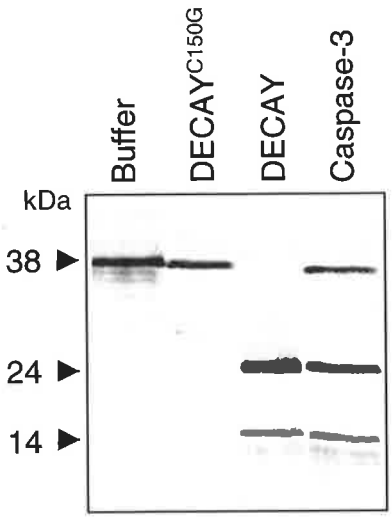
Figure 5.8 DECAY substrate specificity.

(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 ^{35}S-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 DECAY^{C150G} mutant does not cleave PARP.

A


Relative Fluorescence Units
500
450
400
350
300
250
200
150
100
50
0
YVAD-amc
DEVD-amc
VDVAD-amc
Buffer
DECAY^C150G
DECAY
Caspase-2
Caspase-3

B


Buffer
DECAY^C150G
DECAY
Caspase-3
kDa
38
24
14

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 ^{35}S-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*

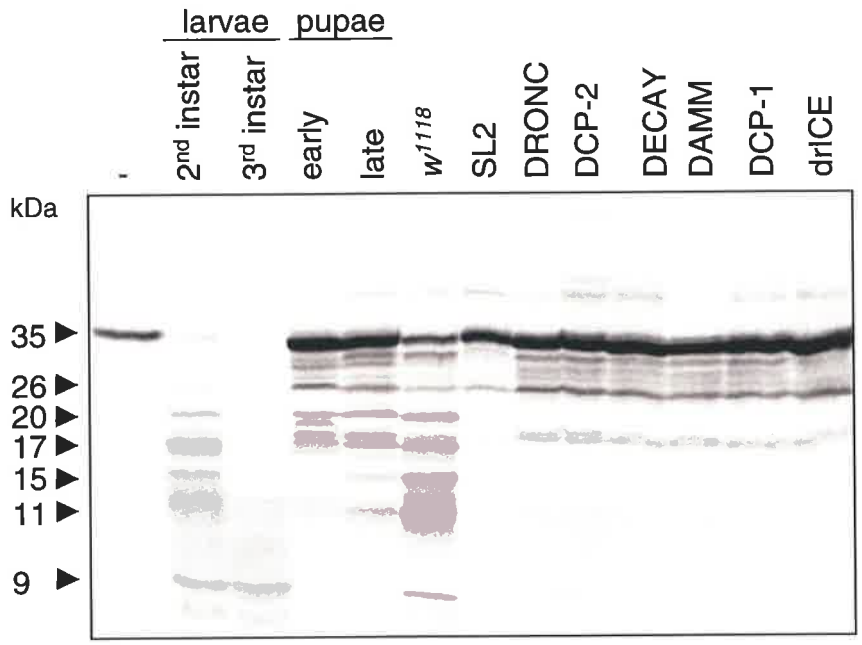
To assess whether DECAY could be processed *in vitro*, ^{35}S-labelled full length DECAY was incubated with various active bacterially expressed caspase lysates and cleavage detected by electrophoresis and autoradiography. As can be seen in Figure 5.9A, DECAY is not processed efficiently by any of the active caspase lysates used. Interestingly, extract prepared from wild type adult flies (w^{1118}) 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).

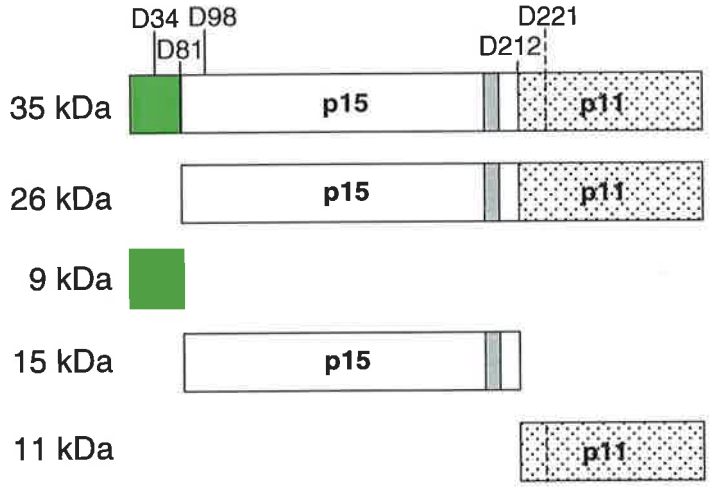
Figure 5.9 *In vitro* processing of DECAY.

(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.

A


larvae
pupae
2nd instar
3rd instar
early
late
w1118
SL2
DRONC
DCP-2
DECAY
DAMM
DCP-1
drICE
-
kDa
35
26
20
17
15
11
9

B

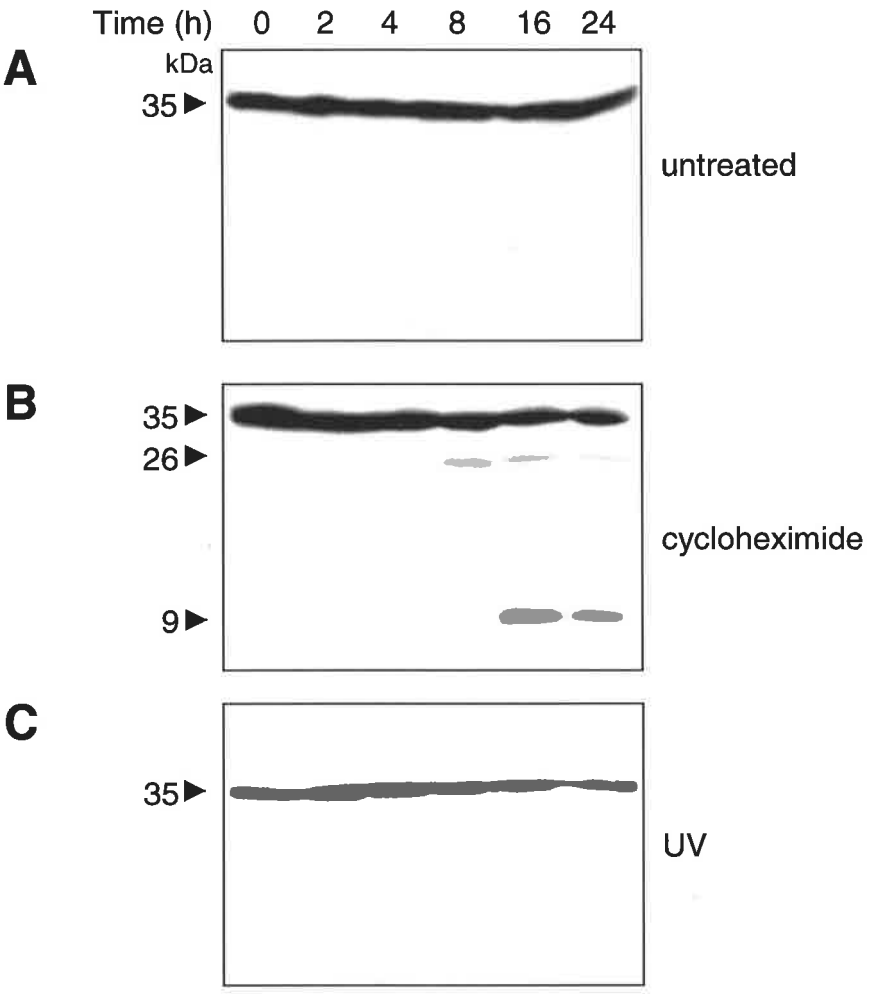

D34
D98
D81
D221
D212
35 kDa
p15
p11
26 kDa
p15
p11
9 kDa
15 kDa
p15
11 kDa
p11

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.


Time (h) 0 2 4 8 16 24
A
kDa
35
untreated
B
35
26
9
cycloheximide
C
35
UV

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, Debcl, 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 $TQTE^{352}$ 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 Dm_0 and DREP-1, ultimately leading to cellular demise (chapter 3, Meier *et al.*, 2000).

The importance of DRONC in *Drosophila* developmental PCD has been demonstrated by RNAi studies in chapter 4. Ablation of *dronc* function resulted in a complete inhibition of PCD in embryos. Additionally, the *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 *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 *dronc* function induces severe hyperplasia of the nervous system. It appears that *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 *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.

Bibliography

Abbott M.K. and Lengyel J.A. (1991) Embryonic head involution and rotation of male terminalia require the *Drosophila* locus *head involution defective*. *Genetics*. **129**: 783-789.

Ahmad M., Srinivasula S.M., Wang L., Litwack G., Fernandes-Alnemri T. and Alnemri E.S. (1997a) *Spodoptera frugiperda* caspase-1, a novel insect death protease that cleaves the nuclear immunophilin FKBP46, is the target of the baculovirus antiapoptotic protein P35. *J. Biol. Chem*. **272**: 1421-1424.

Ahmad M., Srinivasula S.M., Wang L., Talanian R.V., Litwack G., Fernandes-Alnemri T. and Alnemri E.S. (1997b) CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor interacting protein RIP. *Cancer Res*. **57**: 615-619.

Akita K., Ohtsuki T., Nukada Y., Tanimoto T., Namba M., Okura T., Takakura-Yamamoto R., Torigoe K., Gu Y., Su M.S-S., Fujii M., Satoh-Itoh M., Yamamoto K., Kohno K., Ikeda M. and Kurimoto M. (1997) Involvement of caspase-1 and caspase-3 in the production and processing of mature human interleukin-18 in monocytic THP-1 cells. *J. Biol. Chem*. **272**: 26595-26603.

Alnemri E.S., Fernandes-Alnemri T. and Litwack G. (1995) Cloning and expression of four isoforms of human interleukin-1β converting enzyme with different apoptotic activities. *J. Biol. Chem*. **270**: 4312-4317.

Alnemri E.S., Livingston D.J., Nicholson D.W., Salvasen G., Thornberry N.A., Wong W.W. and Yuan J. (1996) Human ICE/CED-3 protease nomenclature. *Cell*. **87**: 171.

Alnemri E.S. (1997) Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases. *J. Cell Biochem*. **64**: 33-42.

Allsopp T.E., Wyatt S., Patterson H.F. and Davies A.M. (1993) The proto-oncogene *bcl-2* can selectively rescue neurotrophic factor-dependent neurones from apoptosis. *Cell.* **73**: 295-307.

Aravind L., Dixit V.M. and Koonin E.V. (2001) Apoptotic molecular machinery: Vastly increased complexity in vertebrates revealed by genome comparisons. *Science.* **291**: 1279-1284.

Artavanis-Tsakonas S., Rand M.D. and Lake R.J. (1999) Notch Signalling: Cell fate control and signal integration in development. *Science.* **284**: 770-776.

Baehrecke E.H. (2000) Steroid regulation of programmed cell death during *Drosophila* development. *Cell Death Differ.* **7**: 1057-1062.

Bakhshi A., Jensen J.P., Goldman P., Wright J.J., McBride O.W., Epstein A.L. and Korsmeyer S.J. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell.* **41**: 899-906.

Baliga B.C. and Kumar S. (2001) Role of Bcl-2 family of proteins in malignancy. *Haem. Oncol.* In press.

Bate M. and Martinez-Arias A. (1993) The development of *Drosophila melanogaster.* *CSHLP.* Vol. II: 905.

Beere H.M., Wolf B.B., Cain K., Masser D.D., Mahboubi A., Kuwana T., Tailor P., Morimoto R.I., Cohen G.M. and Green D.R. (2000) Heat shock protein 70 inhibits apoptosis by preventing recruitment of pro-caspase-9 to the Apaf-1 proteosome. *Nat. Cell Biol.* **2**: 469-475.

Beidler D.R., Tewari M., Friesen P.D., Poirier G. and Dixit V.M. (1995) The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* **270**: 16526-16528.

Bergeron L., Perez G., Macdonald G., Shi L., Sun Y., Jurisicova A., Varmuza S., Latham K.E., Flaws J.A., Salter J.C.M., Hara H., Moskowitz M.A., Li E., Greenberg A., Tilly J.L. and Yuan J. (1998) Defects in regulation of apoptosis in caspase-2 deficient mice. *Nature.* **384**: 368-372.

Bertin J., Nir W.J., Fischer C.M., Tayber O.V., Errada P.R., Grant J.R., Keilty J.J., Gosselin M.L., Robison K.E., Wong G.H., Glucksmann M.A. and DiStefano P.S. (1999) Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J. Biol. Chem.* **274**: 12955-12958.

Birnbaum M.J., Clem R.J. and Miller L.K. (1994) An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a peptide with cys/his sequence motifs. *J. Virol.* **68**: 2521-2528.

Boise L.H., Gonzalez-Garcia M., Postema C.C., Ding L., Lindsten T., Turka L.A., Mao X., Nunez G. and Thompson G.B. (1993) bcl-x., a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell.* **74**: 597-608.

Boldin M.P., Goncharov T.M., Goltsev Y.V. and Wallach D. (1996) Involvement of MACH., a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. *Cell.* **85**: 803-815.

Boyd J.M., Gall G.J., Elangoven B., Houghton A.B., Malstrom S., Avery B.J., Ebb R.G., Subramanian T., Chittenden T., Lutz R.J. and Chinnadurai G. (1995) Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene.* **11**: 1921-1928.

Brachmann C.B., Jassim O.W., Wachsmuth B.D. and Cagan R.L. (2000) The *Drosophila* bcl-2 family member dBorg-1 functions in the apoptotic response to UV-irradiation. *Curr. Biol.* **10**: 547-550.

Brady H.J., Gil-Gomez G., Kirberg J. and Berns A.J. (1996) Bax alpha perturbs T cell development and affects cell cycle entry of T cells. *EMBO J.* **15**: 6991-7001.

Bratton S.B., Walker G., Srinivasula S.M., Sun X-M., Byutterworth M., Alnemri E.S. and Cohen G.M. (2001) Recruitment, activation and retention of caspase-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. *EMBO J.* **20**: 998-1009.

Brodsky M.H., Nordstrom W., Tsang G., Kwan E., Rubin G.M. and Abrams J.M. (2000) *Drosophila* p53 binds a damage response element at the reaper locus. *Cell.* **101**: 103-113.

Bruey J-M., Ducasse C., Bonnaiud P., Ravagnan L., Susin S.A., Diaz-Latoud C., Gurbaxani S., Arrigo A-P., Kroemer G., Solary E. and Garrido C. (2000) Hsp27 negatively regulates cell death by interaction with cytochrome c. *Nat. Cell Biol.* **2**: 645-652.

Bump N.J., Hackett M., Hugunin M., Seshagiri S., Brady K., Chen P., Ferenz C., Franklin S., Ghayur T., Li P., Licari P., Mankovich J., Shi L., Greenberg A.H., Miller L.K. and Wong W.W. (1995) Inhibition of ICE family proteases by baculovirus anti-apoptotic protein p35. *Science.* **269**: 1885-1888.

Burtis K.C., Thummel C.S., Jones C.W., Karim F.D. and Hogness D.S. (1990) The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. *Cell.* **61**: 85-99.

Buszczak M. and Cooley L. (2000) Eggs to die for: cell death during *Drosophila* oogenesis. *Cell Death Differ.* **7**: 1071-1074.

Butt A.J., Harvey N.L., Parasivam G. and Kumar S. (1998) Dimerization and autoprocessing of the Nedd2 (caspase-2) precursor requires both the prodomain and the carboxy terminal regions. *J. Biol. Chem.* **273**: 6763-6768.

Casciola-Rosen L.A., Miller D.K., Anhalt G.J. and Rosen A. (1994) Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* **269**: 30757-30760.

Casciola-Rosen L.A., Anhalt G.J and Rosen A. (1995) DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* **182**: 1625-1634.

Casciola-Rosen L.A., Nicholson D.W., Chong T., Rowan K.R., Thornberry N.A., Miller D.K. and Rosen A. (1996) Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J. Exp. Med.* **183**: 1957-1964.

Cerretti D.P., Kozlosky C.J., Mosley B., Nelson N., Ness K.V., Greenstreet T.A., March C.J., Kronheim S.R., Druck T., Cannizzaro L.A., Huebner K. and Black R.A. (1992) Molecular cloning of the interleukin-1β converting enzyme. *Science.* **256**: 97-100.

Chai J., Du C., Wu J-W., Kyin S., Wang X. and Shi Y. (2000) Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature.* **406**: 855-862.

Chan E.H.Y. and Bonini N.M. (2000) *Drosophila* models of human neurodegenerative disease. *Cell Death Differ.* **7**: 1075-1080.

Chen F., Hersh B.M., Conradt B., Zhou Z., Riemer D., Gruenbaum Y. and Horvitz R.H. (2000) Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death. *Science.* **287**: 1485-1489.

Chen P., Nordstrom H., Gish B. and Abrams J.M. (1996) GRIM, a novel cell death gene in *Drosophila*. *Genes Dev.* **10**: 1773-1782.

Chen P., Rodriguez A., Erskine R., Thach T. and Abrams J.M. (1998) DREDD, a novel effector of the apoptosis activators Rpr, Grim and Hid in *Drosophila*. *Dev. Biol.* **201**: 202-216.

Chinnaiyan A.M., O'Rourke K., Tewari M. and Dixit V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell.* **81**: 505-512.

Chinnaiyan A.M., Orth K., O'Rourke K., Duan H., Poirier G.G. and Dixit V.M. (1996) Molecular ordering of the cell death pathway: Bcl-2 and Bcl-x_L function upstream of the CED-3-like apoptotic proteases. *J. Biol. Chem.* **271**: 4573-4576.

Chinnaiyan A.M., O'Rourke K., Lane B.R. and Dixit V.M. (1997) Interaction of CED-4 with CED-3 and CED-9: A molecular framework for cell death. *Science.* **275**: 1122-1126.

Chittenden T., Harrington E.A., O'Conner R., Flemington C., Lutz R.J., Evan G.I. and Guild B.C. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature.* **374**: 733-736.

Chung S. Gumienny T.L., Hengartner M.O. and Driscoll M. (2000) A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*. *Nat. Cell. Biol.* **2**: 931-937.

Claveria C., Albar J.P., Serrano A., Buesa J.M., Barbero J.L., Martinez-A C. and Torres M. (1998) *Drosophila* grim induces apoptosis in mammalian cells. *EMBO J.* **17**: 7199-7208.

Clem R.J., Fechheimer M. and Miller L.K. (1991) Prevention of apoptosis by a baculovirus gene during infection of insect cells. *Science*. **254**: 1388-1390.

Clem R.J. and Miller L.K. (1994) Control of programmed cell death by the baculovirus genes *p35* and *IAP*. *Mol. Cell Biol.* **14**: 5212-5222.

Colussi P.A., Harvey N.L., Shearwin-Whyatt L.M. and Kumar S. (1998) Conversion of pro-caspase-3 to an autoactivating caspase by fusion to the caspase-2 prodomain. *J. Biol. Chem*. **273**: 26566-26570.

Colussi P.A., Quinn L.M., Huang D.C.S., Coombe M., Read S.H., Richardson H. and Kumar S. (2000) Debcl, a pro-apoptotic Bcl-2 homologue., is a component of the *Drosophila melanogaster* cell death machinery. *J. Cell Biol.* **148**: 703-714.

Conradt B. and Horvitz H.R. (1998) The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2 like protein CED-9. *Cell*. **93**: 519-529.

Cory S. (1995) Regulation of lymphocyte survival by the bcl-2 gene family. *Annu. Rev. Immuno*. **13**: 513-543.

Crook N.E., Clem R.J. and Miller L.K. (1993) An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J. Virol.* **67**: 2168-2174.

Cursio R., Gugenheim J., Ricci J.E., Crenesse D., Rostagno P., Maulon L., Saint-Paul M.C., Ferrua B. and Auberger A.P. (1999) A caspase inhibitor fully protects rats against lethal normothermic liver ischaemia by inhibition of liver apoptosis. *FASEB J.* **13**: 253-261.

Darmon A.J., Nicholson D.W. and Bleackley R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature*. **377**: 446-448.

Davidson F.F. and Stellar H. (1998) Blocking apoptosis prevents blindness in *Drosophila* retinal degeneration mutants. *Nature*. **391**: 587-591.

del Peso L., Gonzalez V.M. and Nunez G. (1998) *Caenorhabditis elegans* EGL-1 disrupts the interaction of CED-9 with CED-4 and promotes CED-3 activation. *J. Biol. Chem*. **273**: 33495-33500.

del Peso L., Gonzalez V.M., Inohara N., Ellis R.E. and Nunez G. (2000) Disruption of the CED-9 CED-4 complex by EGL-1 is a critical step for programmed cell death in *Caenorhabditis elegans*. *J. Biol. Chem*. **275**: 27205-27211.

Deckwerth T.L., Elliot J.L., Knudson C.M., Johnson E.M Jr., Snider W.D. and Korsmeyer S.J. (1996) BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron*. **17**: 401-411.

Deveraux Q.L., Takahashi R., Salvesen G.S. and Reed J.C. (1997) X-linked IAP is a direct inhibitor of cell death proteases. *Nature*. **388**:300-304.

Deveraux Q.L., Roy N., Stennike H.R., Van Arsdale T., Zhou Q., Srinivasula S.M., Alnemri E.S., Salvesen G.S. and Reed J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. *EMBO J.* **17**: 2215-2223.

DiBello P.R., Withers D.A., Bayer C.A., Fristrom J.W and Guild G.M. (1991) The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics*. **129**: 385-397.

Dorstyn L. and Kumar. S. (1997) Differential inhibition of apoptosis by CrmA, P35, IAP and three mammalian IAP homologues mediated by various stimuli. *Cell Death Differ*. **4**: 570-579.

Doumanis J., Quinn L.M., Richardson H. and Kumar S. (2001) STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell Death Differ.* In press.

Du C., Fang M., Li Y., Li L. and Wang X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell.* **102**: 33-42.

Duan H. and Dixit V.M. (1997) RAIDD is a new death adaptor molecule. *Nature.* **385**: 86-90.

Duckett C.S., Nava V.E., Gedrich R.W., Clem R.J., Van Dongen J.L., Gilfillan M.C., Shiels H., Hardwick J.M. and Thompson C.B. (1996) A conserved family of cellular genes related to the baculovirus *iap* gene and encoding apoptosis inhibitors. *EMBO J.* **15**: 2685-2694.

Dushay M.S., Asling B. and Hultmark D. (1996) Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of *Drosophila*. *Proc. Natl. Acad. Sci. USA.* **93**: 10343-10347.

Earnshaw W.C. (1995) Nuclear changes in apoptosis. *Curr. Biol.* **7**: 337-343.

Ellis H.M. and Horvitz H.R. (1986) Genetic control of programmed cell death in the nematode *Caenorhabditis elegans*. *Cell.* **44**: 817-829.

Ellis R.E. and Horvitz H.R. (1991) Two *C. elegans* genes control the programmed deaths of specific cells in the pharynx. *Development.* **112**: 591-603.

Ellis R.E., Yuan J. and Horwitz H.R. (1991) Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* **7**: 663-698.

Elrod-Erickson M., Mishra S. and Schneider D. (2000) Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr. Biol.* **10**: 781-784.

Enari M., Satahira H., Yokoyena H., Otawe K., Iwamatsu A. and Nagata S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*. **391**: 43-50.

Evans E.K., Kuwana T., Strum S.L., Smith J.J., Newmeyer D.D. and Kornbluth S. (1997) Reaper-induced apoptosis in a vertebrate system. *EMBO J.* **16**: 7372-7381.

Faleiro L., Kobayashi R., Fearnhead H. and Lazebnik Y. (1997) Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. *EMBO J.* **16**: 2271-2281.

Farrow S.N., White J.H.M., Martinou I., Raven T., Pun K-T., Grinham C.J., Martinou J-C. and Brown R. (1995) Cloning of a Bcl-2 homologue by interaction with adenovirus E1B 19K. *Nature*. **374**: 731-733.

Fernandes-Alnemri T., Litwack G. and Alnemri E.S. (1994) CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1-β-converting enzyme. *J. Biol. Chem.* **269**: 30761-30764.

Fernandes-Alnemri T., Litwack G. and Alnemri E.S. (1995a) *Mch2*, a new member of the apoptotic *ced-3/Ice* cysteine protease gene family. *Cancer Res.* **55**: 2737-2742.

Fernandes-Alnemri T., Takahashi A., Armstrong R., Krebs J., Fritz L., Tomaselli K.J., Wang L., Yu Z., Croce C.M., Salveson G., Earnshaw W.C., Litwack G. and Alnemri E.S. (1995b) Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res.* **55**: 6045-6052.

Fernandes-Alnemri T., Armstrong R.C., Krebs J., Srinivasula S.M., Wang L., Bullrich F., Fritz L.C., Trapani J.A., Tomaselli K.J., Litwack G. and Alnemri E.S. (1996) *In vitro* activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc. Natl. Acad. Sci. USA.* **93**: 7464-7469.

Flaws J.A., Kuju K., Trbovich A.M., DeSanti A., Tilly K.I., Hirshfield A.N. and Tilly J.L. (1995) Interleukin-1beta-converting enzyme-related proteases (IRPs) and mammalian cell death: Dissociation of IRP-induced oligonucleosomal endonuclease activity from morphological apoptosis in granulosa cells of the ovarian follicle. *Endocrinology.* **136**: 5042-5053.

Fraser A.G., McCarthy N.J. and Evan G.I. (1997) DrICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *EMBO J.* **16**: 6192-6199.

Fraser A.G. and Evan G.I. (1997) Identification of a *Drosophila melanogaster* ICE/ CED-3 related protease, drICE. *EMBO J.* **16**: 2805-2813.

Gagliardini V., Fernandez P-A., Lee R.K.K., Drexler H.C.A., Rotello R.J., Fishman M.C. and Yuan J. (1994) Prevention of vertebrate neuronal death by *crmA* gene. *Science* **263**: 826-828.

Gibson L., Holmgreen S.P., Huang D.C.S., Bernard O., Copeland N.G., Jenkins N.A., Sutherland G.R., Baker E., Adams J.M. and Cory S. (1996) Bcl-w, a novel member of the Bcl-2 family, promotes cell survival. *Oncogene.* **13**: 665-675.

Goltsev Y.V., Kovalenko A.V., Arnold E., Varfolomeev E..E., Brodianski V.M. and Wallach D. (1997) Cash, a novel caspase homologue with death effector domains. *J. Biol. Chem.* **272**: 19641-19645.

Govind S. (1999) Control of development and immunity by rel transcription factors in *Drosophila*. *Oncogene*. **18**: 6875-6887.

Goyal L., McCall K., Agapite J., Hartweig E. and Stellar H. (2000) Induction of apoptosis by *Drosophila reaper*, *hid* and *grim* through inhibition of IAP function. *EMBO J.* **19**: 589-597.

Grether M.E., Abrams J.M., Agapite J., White K. and Stellar H. (1995) The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev.* **9**: 1694-1708.

Gross A., McDonnell M. and Korsmeyer S.J. (1999) Bcl-2 family members and the mitochondria in apoptosis. *Genes Dev.* **13**: 1899-1911.

Gu Y., Sarnecki C., Aldape R.A., Livingston D.J. and Su MS-S. (1995a) Cleavage of poly(ADP-ribose) polymerase by interleukin-1β converting enzyme and its homologs TX and Nedd-2. *J. Biol. Chem.* **270**: 18715-18718.

Gu Y., Wu J., Faucheu C., Lalanne J-L., Diu A., Livingston D. and Su M.S-S. (1995b) Interleukin-1β converting enzyme requires oligomerization for activity of processed forms *in vivo*. *EMBO J.* **14**: 1923-1931.

Gu Y., Kuida K., Tsusui H., Ku G., Hsiao K., Fleming M.A., Hayashi N., Higashino K., Okamura H., Nakanishi K., Kurimoto M., Tanimoto T., Flavell R.A., Sato V., Harding M.W., Livingston D.J. and Su MS-S. (1997) Activation of interferon-γ-inducing factor mediated by interleukin-1β converting enzyme. *Science*. **275**: 206-209.

Haining W.N., Carboy-Newcomb C., Wei C.L. and Stellar H. (1999) The proapoptotic function of *Drosophila* Hid is conserved in mammalian cells. *Proc. Natl. Acad. Sci. USA.* **96**: 4936-4941.

Hakem R., Hakem A., Duncan G.S., Henderson J.T., Woo M., Sorengas M.S., Elia A., de la Pompa J.L., Kagi D., Khoo W., Potter J., Yoshida R., Kaufman S.A., Lowe S.W., Penninger J.M. and Mak T.W. (1998) Differential requirement for caspase-9 in apoptotic pathways *in vivo*. *Cell.* **94**: 339-352.

Hall B.L. and Thummel C.S. (1998) Ultraspiracle is an essential component of the *Drosophila* ecdysone receptor. *Development.* **125**: 4709-4717.

Hammond S.M., Bernstein E., Beach D. and Hannon G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature.* **404**: 293-296.

Han D.K.M., Chaudhary P.M., Wright M.E., Friedman C., Trask B.J., Riedel R.T., Baskin D.G., Schwartz S.M. and Hood L. (1997) MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclxL and initiates cell death. *Proc. Natl. Acad. Sci. USA.* **94**: 11333-11338.

Hara H., Frielander R.M., Gagliardini V., Ayata C., Fink K., Huang Z., Shimizu-Sasamata M., Yuan J. and Moskowitz M.A. (1997) Inhibition of interleukin 1beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. *Proc. Natl. Acad. Sci. USA.* **94**: 2007-2012.

Harvey A.J., Soliman H., Kaiser W.J. and Miller L.K. (1997) Anti- and pro-apoptotic activities of baculovirus and *Drosophila* IAPs in an insect cell line. *Cell Death Differ.* **4**: 733-744.

Harvey N.L., Trapani J.A., Fernandes-Alnemri T., Litwack G., Alnemri E.S. and Kumar S. (1996) Processing of the Nedd2 precursor by ICE-like proteases and granzyme B. *Genes to Cells*

Harvey N.L., Butt A.J. and Kumar S. (1997) Functional activation of Nedd2/Ich-1 (caspase-2) is an early process in apoptosis. *J. Biol. Chem.* **272**: 13134-13139.

Harvey N.L., Daish T., Quinn L.M. Read S.H., Dorstyn L., Richardson H. and Kumar S. (2001) Characterisation of the *Drosophila* caspase, DAMM. *J. Biol. Chem.* In press.

Hawkins C.J., Ekert P.G., Uren A.G., Holmgren S.P. and Vaux D.L. (1998) Anti-apoptotic potential of insect cellular and viral IAPs in mammalian cells. *Cell Death Differ.* **5**: 569-576.

Hawkins C.J., Wang S.L. and Hay B.A. (1999) A cloning method to identify caspases and their regulators in yeast: Identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proc. Natl. Acad. Sci. USA.* **96**: 2885-2890.

Hawkins C.J., Yoo S.J., Peterson E.P., Wang S.L., Vernooy S.Y. and Hay B.A. (2000) The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID and GRIM. *J. Biol. Chem.* **275**: 27084-27093.

Hay B.A., Wolff T. and Rubin G.M. (1994) Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**: 2121-2129.

Hay B.A., Wassarman D.A. and Rubin G.M. (1995) *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell.* **83**: 1253-1262.

Hay B.A. (2000) Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ.* **7**: 1045-1056.

Hedengren M., Asling D., Dushay M.S., Ekengren A.I., Wihlborg M. and Hultmark D. (1999) Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol. Cell.* **4**: 827-837.

Hedgecock E.M., Sulston J.E. and Thomson J.N. (1983) Mutations affecting programmed cell deaths in the nematode *Caenorhabditis elegans*. *Science*. **220**: 1277-1279.

Hengartner M.O., Ellis R.E. and Horvitz H.R. (1992) *C. elegans* gene *ced-9* protects cells from programmed cell death. *Nature*. **356**: 494-499.

Hengartner M.O. and Horvitz H.R. (1994a) *C. elegans* cell survival gene *ced-9* encodes a functional homolog of the mammalian proto-oncogene *bcl-2*. *Cell*. **76**: 665-676.

Hengartner M.O. and Horvitz H.R. (1994b) Programmed cell death in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* **4**: 581-586.

Hengartner M.O. (2000) The biochemistry of apoptosis. *Nature*. **407**: 770-776.

Hetts S.W. (1998) To die or not to die- An overview of apoptosis and its role in disease. *J. Am. Med. Assoc.* **279**: 300-307.

Hirata H., Takahashi A., Kobayashi S., Yonehara S., Sawai H., Okazaki T., Yamamoto K. and Sasada M. (1998) Caspases are activated in a branched protease cascade and control distinct downstream processes in Fas-induced apoptosis. *J. Exp. Med.* **187**: 587-600.

HlaingT., Guo R.F., Dilley K.A., Loussia J.M., Morrish T.A., Shi M.M., Vincenz C. and Ward P. (2000) Molecular cloning and characterisation of DEFCAP-L ans -S two isoforms of a novel member of the mammalian CED-4 family of apoptosis proteins. *J. Biol. Chem.* In press.

Hoffman K.L. and Weeks J. (2001) Role of caspases and mitochondria in the steroid-induced programmed cell death of a motoneuron during metamorphosis. *Dev. Biol.* **229**: 517-536.

Horvitz H.R., Shaham S. and Hengartner M.O. (1994) The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. *Cold Spring Harbor Symp. Quant. Biol.* **27**: 377-385.

Hu S., Vincenz C., Ni J., Gentz R. and Dixit V.M. (1997) I-FLICE, a novel inhibitor of tumour necrosis factor receptor-1 and CD95-induced apoptosis. *J. Biol. Chem.* **272**: 17255-17257.

Hu S. and Yang X. (2000) dFADD, a novel death domain containing adaptor protein for the *Drosophila* caspase DREDD. *J. Biol. Chem.* **275**: 30761-30764.

Hu W-H., Johnson H. and Shu H-B. (2000) Activation of NFkB by FADD, Casper, and caspase-8. *J. Biol. Chem.* **275**: 10838-10844.

Hu Y., Ding L., Spencer DM. and Nunez G. (1998) WD-40 repeat region regulates Apaf-1 self-association and pro-caspase-9 activation. *J. Biol. Chem.* **273**: 33489-33494.

Huang Q., Deveraux Q.L., Maeda S., Salvasen G.S., Stennicke H.R., Hammock B.D. and Reed J.C. (2000) Evolutionary conservation of apoptosis mechanisms: Lepidopteran and baculoviral inhibitor of apoptosis proteins are inhibitors of mammalian caspase-9. *Proc. Natl. Acad. Sci. USA.* **97**: 1427-1432.

Igaki T., Kanuka H., Inohara N., Sawaamoto K., Nunez G., Okano H. and Miura M. (2000) Drob-1, a Drosophila member of the Bcl-2/CED-9 family that promotes cell death. *Proc. Natl. Acad. Sci. USA.* **97**: 662-667.

Imler J-L. and Hoffmann J.A. (2000) Signalling mechanisms in the antimicrobial host defence of *Drosophila*. *Curr. Opin. Microb.* **3**: 16-22.

Inohara N., Koseki T., Hu Y., Chen S. and Nunez G. (1997) CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc. Natl. Acad. Sci. USA.* **94**: 10717-10722.

Inohara N., del Peso L., Koseki T., Chen S. and Nunez G. (1998) RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. *J. Biol. Chem.* **273**: 12296-12230.

Inohara N., Koseki T., Del Peso L., Hu Y., Yee C., Chen S., Carrio R., Merino J., Liu D., Ni J. and Nunez G. (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J. Biol. Chem.* **274**: 145670-145677.

Irmler M., Thome M., Hahne M., Schneider P., Hofmann K., Steiner V., Bodmer J-L., Schroter M., Burns K., Mattmann C., Rimoldi D., French L.E. and Tschopp J. (1997) Inhibition of death receptor signals by cellular FLIP. *Nature.* **388**: 190-195.

Iwahashi H., Eguchi Y., Yasuhara N., Hanafusa T., Matsuzawa Y. and Tsujimoto Y. (1997) Synergistic anti-apoptotic activity between Bcl-2 and SMN implicated in spinal muscular atrophy. *Nature.* **390**: 413-417.

Jackson G.R., Salecker I., Dong X., Yao X., Arnheim N., Faber P.W., MacDonald M.E. and Zipursky S.L. (1998) Polyglutamine-expanded human Huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron.* **21**: 633-642.

James C., Gschmeissner S., Fraser A. and Evan GI. (1997) CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9. *Curr. Biol.* **7**: 246-252.

Jiang C., Baehrecke E.H. and Thummel C.T. (1997) Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development.* **124**: 4673-4683.

Jiang C., Lambin A-F.J., Stellar H. and Thummel C.S. (2000) A steroid-triggered transcriptional hierarchy controls salivary gland cell death during *Drosophila* metamorphosis. *Mol. Cell.* **5**: 445-455.

Jones G., Jones D., Zhou L., Stellar H. and Chu Y. (2000) Deterin, a new inhibitor of apoptosis from *Drosophila melanogaster. J. Biol. Chem.* **275**: 22157-22165

Kaiser W.J., Vucic D. and Miller L.K. (1998) The *Drosophila* inhibitor of apoptosis DIAP1 suppresses cell death induced by the caspase drICE. *FEBS Letters.* **440**: 243-248

Kang S-J., Wang S., Hara H., Peterson E.P., Namura S., Amin-Hanjani S., Huang Z., Srinivasan A., Tomaselli K.J., Thornberry N.A., Moskowitz M.A. and Yuan J. (2000) Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. *J. Cell Biol.* **149**: 613-622.

Kanuka H., Hisahara S., Sawamoto K., Shoji S-I., Okano H. and Miura M. (1999a) Proapoptotic activity of *Caenorhabditis elegans* CED-4 protein in *Drosophila*: Implicated mechanisms for caspase activation. *Proc. Natl. Acad. Sci. USA.* **96**: 145-150.

Kanuka H., Sawamoto K., Inohara N., Matsuno K., Okano H. and Miura M. (1999b) Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Mol. Cell.* **4**: 757-769.

Kaufmann S.H., Desnoyers S., Ottaviano Y., Davidson N.E. and Poirier G.G. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.* **53**: 3976-3985.

Kaye F.J., Modi S., Ivanovska I., Koonin E.V., Thress K., Kubo A., Kornbluth S. and Rose M.D. (2000) A family of ubiquitin-like proteins binds the ATPase domain of Hsp70-like Stch. *FEBS Letters*. **467**: 348-355.

Kerr J.F.R., Wyllie A.H. and Currie A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Can.* **26**: 239-257.

Kinoshita M., Tomimoto H., Kinoshita A., Kumar S. and Noda M. (1997) Up-regulation of the Nedd2 gene encoding an ICE/Ced-3-like cysteine protease in the gerbil brain after transient global ischaemia. *J. Cereb. Blood Flow Metab.* **17**: 507-514.

Klingsensmith J., Noll E. and Perrimon N. (1989) The segment polarity phenotype of *Drosophila* involves different tendencies toward transformation and cell death. *Dev. Biol.* **134**: 130-145.

Kluck R.M., Bossy-Wetzel E., Green D.R. and Newmeyer D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science.* **275**: 1132-1136.

Kluck R.M., Esposti M.D., Pekins G., Renken C., Kuwana T., Bossy-Wetzel E., Goldberg M., Allen T., Barber M.J., Green D.R. and Newmeyer D.D. (1999) The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J. Cell Biol.* **147**: 809-822.

Knudsen C.M., Tung KS., Tourtellotte WG., Brown GA. and Korsmeyer SJ. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. **270**: 96-99.

Komiyama T., Ray C.A., Pickup D.J., Howard A.D., Thornberry N.A., Peterson E.P. and Salvesen G. (1994) Inhibition of interleukin-1β converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J. Biol. Chem.* **269**: 19331-19337.

Kondo T., Yokokura T. and Nagata S. (1997) Activation of distinct caspase-like proteases by Fas and Reaper in *Drosophila* cells. *Proc. Natl. Acad. Sci. USA.* **94**: 11951-11956.

Korsmeyer S.J. (1992) *Bcl-2* initiates a new category of oncogenes: Regulators of cell death. *Blood.* **80**: 879-886.

Krajewski S., Mai J.K., Krajewska M., Sikorska M., Mossakowski M.J. and Reed J.C. (1995) Upregulation of Bax levels in neurons following cerebral ischaemia. *J. Neurosci.* **15**: 6364-6376.

Kroemer G. and Reed J.C. (2000) Mitochondrial control of cell death. *Nat. Med.* **6**: 513-519.

Kuida K., Lippke J.A., Ku G., Harding M.W., Livingston D.J., Su MS-S. and Flavell R.A. (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1beta converting enzyme. *Science.* **267**: 2000-2003.

Kuida K., Zheng T.S., Na S., Kuan C-Y., Yang D., Karasuyama H., Rakic P. and Flavell R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. *Nature.* **384**: 368-372.

Kuida K., Haydar T.F., Kuan C-Y., Gu Y., Taya C., Karasuyama H., Su MS-S., Rakic P. and Flavell R.A. (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase-9. *Cell.* **94**: 325-352.

Kumar S., Tomooka Y. and Noda M. (1992) Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem. Biophys. Res. Commun.* **185**: 1155-1161.

Kumar S., Kinoshita M., Noda M., Copeland N.G. and Jenkins N.A. (1994) Induction of apoptosis by the mouse *Nedd2* gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene *ced-3* and the mammalian IL-1β converting enzyme. *Genes Dev.* **8**: 1613-1626.

Kumar S. (1995) Inhibition of apoptosis by the expression of antisense *Nedd2*. *FEBS Letters*. **368**: 69-72.

Kumar S. and Harvey N.L. (1995) Role of multiple cellular proteases in the execution of programmed cell death. *FEBS Letters*. **375**: 169-173.

Kumar S. and Lavin M.F. (1996) The ICE family of cysteine proteases as effectors of cell death. *Cell Death Differ*. **3**: 255-267.

Kumar S., Kinoshita M., Dorstyn L. and Noda M. (1997) Origin, expression and possible functions of the two alternatively spliced forms of the mouse *Nedd2* mRNA. *Cell Death Diff.* **4**: 378-387.

Kumar S. (1999) Mechanisms mediating caspase activation in cell death. *Cell Death Differ.* **6**: 1060-1066.

Kumar S. and Colussi P.A. (1999) Prodomains-adaptors-oligomerization: the pursuit of caspase activation in apoptosis. *Trends Biochem. Sci.* **24**: 1-4.

Kumar S. and Doumanis J. (2000) The fly caspases. *Cell death and Differ.* **7**: 1039-1044.

Kurada P. and White K. (1998) Ras promotes cell survival in *Drosophila melanogaster* by downregulating *hid* expression. *Cell*. **95**: 319-329.

La Count D.J., Hanson S.F., Schneider C.L. and Friesen P.D. (2000) Caspase inhibitor p35 and inhibitor of apoptosis OpIAP block *in vivo* proteolytic activation of an effector caspase at different steps. *J. Biol. Chem*. **275**: 15657-15664.

Lazebnik Y.A., Kaufmann S.H., Desnoyers S., Poirier G.G. and Earnshaw W.C. (1994) Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature*. **371**: 346-347.

Lee C-Y. and Baehrecke E.H. (2000) Genetic regulation of programmed cell death in *Drosophila. Cell Res*. **10**: 193-204.

Lee C-Y., Wendel D.P., Reid P., Lam G., Thummel C.S. and Baehrecke E.H. (2000) *E93* directs steroid-triggered programmed cell death in *Drosophila. Mol. Cell*. **6**: 433-443.

Lee D., Long S.A., Adams J.L., Chan G., Vaida K.S., Francis T.A., Kikly K., Winkler J.D., Sung C-M., Debouk C., Richardson S., Levy M.A., DeWolf WE Jnr., Keller P.M., Tomaszek T., Head M.S., Ryan M.D., Haltiwanger R.C., Liang P-H., Janson C.A., McDevitt P.J., Johanson K., Concha N.O., Chan W., Abdel-Meguid S.S., Badger A.M., Lark M.W., Nadeau D.P., Suva L.J., Gowen M. and Nuttall M.E. (2000) Potent and selective nonpeptide inhibitors of caspase-3 and -7 inhibit apoptosis and maintain cell functionality. *J. Biol. Chem*. **275**: 16007-16014.

Leulier F., Rodriguez A., Khush R.S., Abrams J.M. and Lemaitre B. (2000) The *Drosophila* caspase Dredd is required to resist gram negative bacterial infection. *EMBO reports*. **1**: 353-358.

Li F., Ambrosini G., Chu E.Y., Plescia J., Tognin S., Marchisio P.C. and Alteri D.C. (1998) Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*. **396**: 580-584.

Li F., Ackermann E.J., Bennett C.F., Rothermel A.L., Plescia J., Tognin S., Villa A., Marchisio P.C. and Altieri D.C. (1999) Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nat. Cell. Biol.* **1**: 461-466.

Li H., Zhu H., Xu C.J. and Yuan J. (1998) Cleavage of BID by caspase-8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. **94**: 491-501.

Li K., Li Y., Shelton J.M., Richardson J.A., Spencer E., Chen ZJ., Wang X. and Williams R.S. (2000a) Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis. *Cell*. **101**: 389-399.

Li M., Ona V.O., Guegan C., Chen M., Jackson-Lewis V., Andrews L.J., Olszewski A.J., Stieg P.E., Lee J-P., Przedborski S. and Friedlander R.M. (2000b) Functional role of caspase-1 and caspase-3 in ALS transgenic mouse model. *Science*. **288**: 335-339.

Li P., Allen H., Banerjee S., Franklin S., Herzog L., Johnston C., McDowell J., Paskind M., Rodman L., Salfeld J., Towne E., Tracey D., Wardwell S., Wei F-Y., Wong W., Kamen R. and Seshadri T. (1995) Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxin shock. *Cell*. **80**: 401-411.

Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M., Alnemri E.S. and Wang X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates the apoptotic protease cascade. *Cell*. **91**: 479-489.

Lindsten T., Ross A.J., King A., Zong W., Rathmell J.C., Shiels H.A., Ulrich E., Waymire K.G., Mahar P., Frauwirth K., Chen Y., Wei M., Eng V.M., Adelman D.M., Celeste Simon M., Ma A., Golden J.A., Evan G., Korsmeyer S.J., MacGregor G.R. and Thompson C.B. (2000) The combined functions of pro-apoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. *Mol. Cell.* **6**: 1389-1399.

Lisi S., Mazzon I. and White K. (2000) Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. *Genetics*. 154: 669-678.

Liston P., Roy N., Tamai K., Lefebvre C., Baird S., Cherton-Horvat G., Farahani R., McLean M., Ikeda J-E., MacKenzie A. and Korneluk R.G. (1996) Suppression of apoptosis in mammalian cells by NAIP and related family of IAP genes. *Nature*. **379**: 349-353.

Liu Q.A. and Hengartner M.O. (1998) Candidate adaptor protein CED-6 promotes the engulfment of apoptotic cells in *C. elegans*. *Cell*. **93**: 961-972.

Liu Q.A. and Hengartner M.O. (1999a) Human CED-6 encodes a functional homologue of the *Caenorhabditis elegans* engulfment protein CED-6. *Curr. Biol.* **9**: 1347-1350.

Liu Q.A. and Hengartner M.O. (1999b) The molecular mechanism of programmed cell death in *C. elegans*. *Ann. N. Y. Acad. Sci.* 887:92-104.

Liu X., Kim C.K., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell free extracts: requirement for dATP and cytochrome c. *Cell*. **86**: 147-157.

Liu X., Zou H., Slaughter C. and Wang X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*. **86**: 147-157.

Locksley R.M., Killeen N. and Lenardo M.J. (2001) The TNF and TNF-receptor superfamilies: Integrating mammalian biology. *Cell*. **104**: 487-501.

Majno G. and Joris I. (1995) Apoptosis, oncosis and necrosis. An overview of cell death. *Am. J. Pathol*. **146**: 3-15.

Maniatis T., Sambrook J. and Fritsch E.F. (1989) Molecular cloning: A laboratory manual. (2^{nd} ed)

Manji G.A. and Friesen P.D. (2001) Apoptosis in motion: An apical, P35-insensitive caspase mediates programmed cell death in insect cells. *J. Biol. Chem*. In press.

Margolin N., Raybuck S.A., Wilson K.P., Chen W., Fox T., Gu Y. and Livingston D.J. (1997) Substrate and inhibitor specificity of interleukin-1β-converting enzyme and related caspases. *J. Biol. Chem*. **272**: 7223-7228.

Martinou I., Fernandez P.A., Missotten M., White E., Allet B., Sadoul A. and Martinou. J.C. (1995) Viral proteins E1B19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. *J. Cell Biol.* **128**: 201-208.

Martins L.M., Kottke T., Mesner P.W., Basi G.S., Sinha S., Frigon N. Jr., Tatar E., Tung J.S., Bryant K., Takahashi A., Svingen P.A., Madden B.J., McCormick D.J., Earnshaw W.C. and Kaufmann S.H. (1997) Activation of multiple interleukin-1β converting enzyme homologues in cytosol and nuclei of HL-60 cells during etoposide-induced apoptosis. *J. Biol. Chem*. **272**: 7421-7430.

McCall K. and Stellar H. (1998) Requirement for DCP-1 caspase during *Drosophila* oogenesis. *Science*. **279**: 230-234.

McCarthy J.V. and Dixit V.M. (1998) Apoptosis induced by *Drosophila* reaper and grim in a human system. *J. Biol. Chem.* 273: 24009-24015.

McCarthy J.V., Ni J. and Dixit V.M. (1998) RIP2 is a novel NF-kappaB-activating and death-inducing kinase. *J. Biol. Chem.* **273**: 16968-16975.

Meier P., Silke J., Leevers S.J. and Evan G.I. (2000) The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J.* **19**: 598-611.

Michaelidis T.M., Sendtner M., Cooper J.D., Airaksinen M.S., Holtmann B., Meyer M. and Thoenen H. (1996) Inactivation of *bcl-2* results in progressive degeneration of motorneurons, sympathetic and sensory neurons during early postnatal development. *Neuron.* **17**: 75-89.

Miller D.T. and Cagan R.L. (1998) Local induction of programmed cell death in the developing *Drosophila* retina. *Development.* **125**: 2327-2335.

Minn A.J., Velez P., Schendel S.L., Liang H., Muchmore S.W., Fesik S.W., Fill M. and Thompson C.B. (1997) Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature.* **385**: 353-357.

Minn A.J., Kettlun C.S., Liang H., Kelekar A., Van der Heiden M.G., Chang B.S., Fesik S.W., Fill M. and Thompson C.B. (1999) Bcl-x_L regulates apoptosis by heterodimerisation dependent and independent mechanisms. *EMBO J.* **18**: 632-643.

Mittl P.R., Di Marco S., Krebs J.F., Bai X., Karanewski D.S., Priestle J.P., Tomaselli K.J. and Grutter M.G. (1997) Structure of recombinant CPP32 in a complex with the tetrapeptide acety-Asp-Val-Ala-Asp fluoromethyl ketone. *J. Biol. Chem.* **272**: 6539-6547.

Miura M., Zhu H., Rotello R., Hartwieg E.A. and Yuan J. (1993) Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammlian homolog of the *C. elegans* cell death gene *ced-3*. *Cell*. **75**: 653-660.

Miura M., Friedlander R.M. and Yuan J. (1995) Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway. *Proc. Natl. Acad. Sci. USA*. **92**: 8318-8322.

Motoyama N., Wang F., Roth K.A., Sawa H., Nakayama K., Negishi I., Senju S., Zhang Q., Fujii S. and Loh D.Y. (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*. **267**: 1506-1510.

Muzio M., Chinnaiyan A.M., Kischel F.C., O'Rourke K., Shevchenko A., Scaffidi C., Bretz J.D., Zhang M., Ni J., Gentz R., Mann M., Krammer P.H., Peter M.E. and Dixit V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signalling complex (DISC). *Cell*. **85**: 817-827.

Nagata S. and Golstein P. (1995) The Fas death factor. *Science*. **267**: 1449-1456.

Nagata S. (2000) Steering anti-cancer drugs away from the TRAIL. *Nat. Med*. **6**: 502-503.

Nakayama K., Nakayama K., Negishi I., Kuida K., Sawa H. and Loh D.Y. (1994) Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proc. Natl. Acad. Sci. USA*. **91**: 3700-3704.

Nassif C., Daniel A., Lengyel J.A. and Hartenstein V. (1998) The role of morphogenetic cell death during *Drosophila* embryonic head development. *Dev Biol*. **197**: 170-186

Nelson Chau B., Cheng E.H-Y., Kerr D.A. and Hardwick J.M. (2000) Aven, a novel inhibitor of caspase activation binds BclxL and Apaf-1. *Mol. Cell*. **6**: 31-40.

Nguyen M., Branton P.E., Walton P.A., Oltvai Z.N., Korsmeyer S.J. and Shore G.C. (1994) Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. *J. Biol. Chem.* **269**: 16521-16524.

Nicholson D.W., Ali A., Thornberry N.A., Vaillancourt J.P., Ding C.K., Gallant M., Gareau Y., Griffin P.R., Labelle M., Lazebnik Y.A., Munday N.A., Raju S.M., Smulson M.E., Yamin T.T., Yu V.L. and Miller D.K. (1995) Identification and inhibition of the ICE/Ced-3 protease necessary for mammalian apoptosis. *Nature*. **376**: 37-43.

Nicholson D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ*. **6**: 1028-1042.

Nicholson D.W. (2000) From bench to clinic with apoptosis-based therapeutic agents. *Nat. Rev.*. **407**: 810-816.

Niwa H., Yamamura K. and Miyazaki J. (1991) Efficient selection of high-expression transfectants with a novel eukaryotic vector. *Gene*. **108**: 193-200.

Nordstrom W., Chen P., Stellar H. and Abrams J.M. (1996) Activation of the reaper gene during ectopic cell killing in *Drosophila*. *Dev. Biol.* **180**: 213-226.

Oeda E., Oka Y., Miyazono K. and Kawabata M. (1998) Interaction of *Drosophila* inhibitors of apoptosis with thick veins, a type I serine/threonine kinase receptor for decapentaplegic. *J. Biol. Chem.* **273**: 9353-9356.

Ogasawari J., Wantanabe-Fukunaga R., Adachi M., Matsuzawa A., Kasugai T., Kitamura Y., Itoh N., Suda T. and Nagata S. (1993) Lethal effect of the anti-Fas antibody in mice. *Nature*. **364**: 806-809.

Ogura Y., Inohara N., Benito A., Chen F.F., Yamaoka S. and Nunez G. (2000) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NFκB. *J. Biol. Chem.* **276**: 4812-4818.

Oltvai Z.N., Milliman C.L. and Korsmeyer S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homologue, Bax, that accelerates programmed cell death. *Cell.* **74**: 609-619.

Palumbo G.J., Pickup D.J., Fredrickson T.N., McIntyre L.J. and Buller R.M. (1989) Inhibition of an inflammatory response is mediated by a 38-kDa protein of cowpox virus. *Virology.* **171**: 262-273.

Pandey P., Saleh A., Nakazawa A., Kumar S., Srinivasula SM., Kumar V., Weichselbaum R., Nalin C., Alnemri E.S., Kufe D. and Kharbanda S. (2000) Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of pro-caspase-9 by heat shock protein 90. *EMBO J.* **19**: 4310-4322.

Petritsch C., Wang S.W., Jan L.Y. and Jan Y.N. (2000a) The divergent caspase DRONC interacts with Numb protein and is capable of affecting sense organ cell fate. *Ann. Dros. Res. Conf. 41*; abstract.

Petritsch C., Wang S.W. and Jan L.Y. (2000b) The divergent caspase DRONC processes Notch and regulates neurogenesis. *Mol. Biol. Cell. (suppl.)* 11: 290A-290A

Polverino A.J. and Patterson S.D. (1997) Selective activation of caspases during apoptotic induction in HL-60 cells. *J. Biol. Chem.* **272**: 7013-7021.

Pronk G.J., Ramer K., Amiri P. and Williams L.T. (1996) Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. *Science.* **271**: 808-810.

Purring-Koch C. and McLendon G. (2000) Cytochrome c binding to Apaf-1: The effects of dATP and ionic strength. *Proc. Natl. Acad. Sci. USA*. **97**: 11928-11931.

Qin H., Srinivasula S.M., Wu G., Fernandes-Alnemri T., Alnemri E.S. and Shi Y. (1999) Structural basis of procaspase-9 recruitment by the apoptotic protease-activating factor-1. *Nature*. **399**: 549-557.

Rabizadeh S., LaCount D.J., Friesen P.D. and Bredesen D.E. (1993) Expression of the baculovirus *p35* gene inhibits mammalian neural cell death. *J. Neurochem*. **61**: 2318-2321.

Ramage P., Cheneval D., Chvei M., Graff P., Hemmig R., Heng R., Kocher H.P., Mackenzie A., Memmert K., Revesz L. and Wishart W. (1995) Expression, refolding, and autocatalytic proteolytic processing of the interleukin-1β-converting enzyme precursor. *J. Biol. Chem*. **270**: 9378-9383.

Rampino N., Yamamoto H., Ionov Y., Li Y., Sawai H., Reed J.C. and Perucho M. (1997) Somatic frameshift mutations in the BAX gene in colon cancers of the microsattelite mutator phenotype. *Science*. **275**: 967-969.

Rano T.A., Timkey T., Peterson E.P., Rotonda J., Nicholson D.W., Becker J.W., Chapman K.T. and Thornberry N.A. (1997) A combinatorial approach for determining protease specificities: application to interleukin-1β converting enzyme (ICE). *Chem. Biol*. **4**: 149-155.

Rasper D.M., Vaillancourt J.P., Hadano S., Houtzager V.M., Seiden I., Keen S.L., Tawa P., Xanthoudakis S., Nasir J., Martindale D., Koop B.F., Peterson E.P., Thornberry N.A., Huang J., MacPherson D.P., Black S.C., Hornung F., Lenardo M.J., Hayden M.R., Roy S. and Nicholson D.W. (1998) Ursurpin, a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD95 (Fas/Apo-1) receptor complex. *Cell Death Differ*. **5**: 271-288.

Ray C.A., Black R.A., Kronheim S.R., Greenstreet T.A., Sleath P.R., Salvesen G.S. and Pickup D.J. (1992) Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1β converting enzyme. *Cell.* **69**: 597-604.

Reddien P.W. and Horvitz H.R. (2000) CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in *Caenorhabditis elegans*. *Nat. Cell Biol.* **2**: 131-136.

Reed J.C. and Kroemer G. (2000) Mechanisms of mitochondrial membrane permeabilisation. *Cell Death Differ.* **7**: 1145

Richardson H., O'Keefe L.V., Marty T. and Saint R. (1995) Ectopic cyclin E expression induces premature entry into S phase and disrupts pattern formation in the *Drosophila* eye imaginal disc. *Development.* 121: 3371-3379.

Robinow S., Talbot W.S., Hogness D.S. and Truman J.W. (1993) Programmed cell death in the *Drosophila* central nervous system is ecdysone regulated and coupled with a specific ecdysone receptor isoform. *Development.* **119**: 1251-1259.

Robinow S., Draizen T.A. and Truman J.W. (1997) Genes that induce apoptosis: transcription regulation in identified, doomed neurons of the *Drosophila* CNS. *Dev. Biol.* **190**: 206-213.

Rodriguez A., Oliver H., Zou H., Chen P., Wang X. and Abrams J.M. (1999) Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol.* **1**: 272-279.

Rodriguez I., Mataura K., Ody C., Nagata S. and Vassalli P. (1996) Systemic injection of a tetrapeptide inhibits the intracellular activation of CPP32-like proteases *in vivo* and fully protects mice against Fas-mediated fulminent liver destruction and death. *J. Exp. Med.* **184**: 2067-2072.

Rothe R., Pan M.G., Henzel W.J., Ayres T.M. and Goeddel D.V. (1995) The TNFR2-TRAF signalling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell.* **83**: 1243-1252.

Rotonda J., Nicholson D.W., Fazil K.M., Gallant M., Gareau Y., Labelle M., Peterson EP., Rasper DM., Ruel R., Vaillancourt JP., Thornberry NA. and Becker JW. (1996) The three dimensional structure of apopain/CPP32, a key mediator of apoptosis. *Nat. Struct. Biol.* **3**: 619-625.

Roy N., Mahadevan M.S., McLean M., Shutler G., Yaraghi Z., Ferahani R., Baird S., Besner-Johnston A., Lefebvre C., Kang X., Salih M., Aubry H., Tamai K., Guan X., Ioannou P., Crawford TO., de Jong PJ., Surh L., Ikeda J., Korneluk R.G. and MacKenzie A. (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell.* **80**: 167-178.

Roy N., Deveraux Q.L., Takahashi R., Salvasen G.S. and Reed J.C. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* **16**: 6914-6925.

Rubin G.M., Yandell M.D., Wortman J.R., Gabor Miklos G.L., Nelson C.R., Hariharan I.K., Fortini M.E., Li P.W., Apweiler R., Fleischmann W., Cherry J.M., Henikoff S., Skupski M.P., Misra S., Ashburner M., Birney E., Boguski M.S., Brody T., Brokstein P., Celniker S.E., Chervitz S.A., Coates D., Cravchik A., Gabrielian A., Galle R.F., Gelbart W.M., George R.A., Goldstein L.S.B., Gong F., Guan P., Harris N.L., Hay B.A., Hoskins R.A., Li J., Li Z., Hynes R.O., Jones S.J.M., Kuehl P.M., Lemaitre B., Littleton J.T., Morrison D.K., Mungall C., O'Farrell P.H., Pickeral O.K., Shue C., Vosshall L.B., Zhang J., Zhao Q., Zheng X.H., Zhong F., Zhong W., Gibbs R., Venter J.C., Adams M.D. and Lewis S. (2000) Comparative genomics of the eukaryotes. *Science.* **287**: 2204-2218.

Sakahira H., Enari M. and Nagata S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature.* **391**:96-99.

Saleh A., Srinivasula S.M., Acharya S., Fishel R. and Alnemri E.S. (1999) Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J. Biol. Chem.* **274**: 17941-17945.

Saleh A., Srinivasula S.M., Balkir L., Robbins P.D. and Alnemri E.S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat. Cell Biol.* **2**: 476-483.

Segraves W.A. and Hogness D.S. (1990) The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two members of the steroid receptor superfamily. *Genes Dev.* **4**: 204-219.

Shaham S. and Horvitz H.R. (1996) An alternatively spliced *C. elegans ced-4* RNA encodes a novel cell death inhibitor. *Cell.* **86**: 201-208.

Shaham S. (1998) Identification of multiple *Caenorhabditis elegans* caspases and their potential roles in proteolytic cascades. *J. Biol. Chem.* **273**: 35109-35117.

Sharp P.A. (1999) RNAi and double-strand RNA. *Genes Dev.* **13**: 139-141.

Shearwin-Whyatt L.M. and Kumar S. (1999) Caspases in developmental cell death. *IUBMB Life.* **48**: 143-150.

Shearwin-Whyatt L.M., Harvey N.L. and Kumar S. (2000) Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. *Cell Death Differ.* **7**: 155-165.

Shen Y. and Shenk T.E. (1995) Viruses and apoptosis. *Genes Dev.* **5**: 105-111.

Shimizu S., Eguchi Y., Kamiike W., Matsuda H. and Tsujimoto Y. (1996a) Bcl-2 expression prevents activation of the ICE protease cascade. *Oncogene* **12**: 2251-2257.

Shimizu S., Eguchi Y., Kamiike W., Waguri S., Uchiyama Y., Matsuda H. and Tsujimoto Y. (1996b) Bcl-2 blocks loss of mitochondria membrane potential while ICE inhibitors act at a different step during inhibition of death induced by respiratory chain inhibitors. *Oncogene*. **13**. 21-29.

Shu H.B., Halpin D.R. and Goeddel D.V. (1997) Casper is a FADD-and caspase-related inducer of apoptosis. *Immunity*. **6**: 751-763.

Silverman N., Zhou R., Stavens S., Pandey N., Hultmark D. and Maniatis T. (2000) A *Drosophila* IκB kinase complex required for Relish cleavage and antibacterial immunity. *Genes Dev*. **14**: 2461-2471.

Smits E., Van Criekinge W., Plaetinck G. and Bogaert T. (1999) The human homologue of *Caenorhabditis elegans* CED-6 specifically promotes phagocytosis of apoptotic cells. *Curr. Biol*. **9**: 1351-1354.

Song Q., Lees-Miller S.P., Kumar S., Zhang N., Chan D.W., Smith G.C.M., Jackson S.P., Alnemri E.S., Litwack G., Khanna K.K. and Lavin M.F. (1996) DNA-dependant protein kinase catalytic subunit: A target for an ICE-like protease in apoptosis. *EMBO. J* **15**: 3238-3246.

Song Z., McCall K. and Stellar H. (1997) DCP-1, a *Drosophila* cell death protease essential for development. *Science*. **275**: 536-540.

Song Z., Guan B., Bergman A., Nicholson D.W., Thornberry N.A., Peterson E.P. and Stellar H. (2000) Biochemical and genetic interactions between *Drosophila* caspases and the proapoptotic genes *rpr*, *hid* and *grim*. *Mol. Cell. Biol*. **20**: 2907-2914.

Sonnenfeld M. and Jacobs R. (1995) Apoptosis of the midline glia during *Drosophila* embryogenesis: a correlation with axon contact. *Development*. **121**: 569-578.

Srinivasula S.M., Fernandes-Alnemri T., Zangrilli J., Robertson N., Armstrong R.C., Wang L., Trapani J.A., Tomaselli K.J., Litwack G. and Alnemri E.S. (1996) The ced-3/ interleukin-1β converting enzyme like homolog Mch6 and the lamin cleaving enzyme Mch2α are the substrates for the apoptotic mediator CPP32. *J. Biol. Chem.* **271**: 27099-27106.

Srinivasula S.M., Ahmad M., Ottilie S., Bullrich F., Banks S., Wang Y., Fernandes-Alnemri T., Croce C.M., Litwack G., Tomaselli K.J., Armstrong R.C. and Alnemri E.S. (1997) FLAME-1, a novel FADD-like anti-apoptotic molecule that regulates Fas/TNFR-1-induced apoptosis. *J. Biol. Chem.* 272: 18542-18545.

Srinivasula S.M., Ahmad M., Fernandes-Alnemri T. and Alnemri E.S. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerisation. *Mol. Cell.* **1**: 949-957.

Srinivasula S.M., Ahmad M., Guo Y., Zhan Y., Lazebnik Y., Fernandes-Alnemri T. and Alnemri E.S. (1999) Identification of an endogenous dominant negative short isoform of caspase-9 that can regulate apoptosis. *Cancer Res.* **59**: 999-1002.

Srinivasula S.M., Datta P., Fan X-J., Fernandes-Alnemri T., Huang Z. and Alnemri E.S. (2000) Molecular determinants of the caspase-promoting activity of Smac/DIABLO and its role in the death receptor pathway. *J. Biol. Chem.* **275**: 36152-36157.

Srinivasula S.M., Hegde R., Saleh A., Datta P., Shiozaki E., Chai J., Lee R-A., Robbins P.D., Fernandes-Alnemri T., Shi Y. and Alnemri E.S. (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/Diablo regulates caspase activity and apoptosis. *Nature.* **410**: 112-116.

Stanfield G.M. and Horvitz H.R. (2000) The *ced-8* gene controls the timing of programmed cell deaths in *C. elegans*. *Mol. Cell.* **5**: 423-433.

Stellar H. (1995) Mechanisms and genes of cellular suicide. *Science*. **267**: 1445-1449.

Stoven S., Ando I., Kadalayi L., Engstrom Y. and Hultmark D. (2000) Activation of *Drosophila* NFκB factor Relish by rapid endoproteolytic cleavage. *EMBO reports*. **1**: 347-352.

Strasser A., O'Connor L. and Dixit V.M. (2000) Apoptosis signalling. *Ann. Rev. Biochem.* **69**: 217-245.

Sugimoto A., Friesen P.D. and Rothman J.H. (1994) Baculovirus p35 prevents developmentally programmed cell death and rescues a *ced-9* mutant in the nematode *Caenorhabditis elegans*. *EMBO J.* **13**: 2023-2028.

Sullivan W., Ashburner M. and Hawley R.S. (2000) *Drosophila* protocols. *CSHLP.*

Sulston J.E., Schierenberg E., White J.G. and Thompson J.N. (1983) The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**: 64-119.

Susin S.A., Zamzami N., Castedo M., Hirsch T., Marchetti P., Macho A., Daugas E., Geuskens M. and Kroemer G. (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med.* **184**: 1331-1341.

Susin S.A., Lorenzo H.K., Zamzami M., Marzo I., Brenner C., Larochette N., Prevost M.C., Alzari P.M. and Kroemer G. (1999) Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J. Exp. Med.* **189**: 381-394.

Takahashi A., Alnemri E.S., Lazebnik Y.A., Fernandes-Alnemri T., Litwack G., Moir R.D., Goldman R.D., Poirer G.G., Kaufmann S.H. and Earnshaw W.C. (1996) Cleavage of lamin A by Mch2 alpha but not CPP32: multiple interleukin-1-beta-converting enzyme-related proteases with distinct substrate recognition properties are active in apoptosis. *Proc. Natl. Acad. Sci. USA.* **93**: 8395-8400.

Takahashi R., Deveraux Q., Tamm I., Welsh K., Assamunt N., Salvasen G.S. and Reed J.C. (1998) A single BIR-domain of XIAP is sufficient for inhibiting caspases. *J. Biol. Chem.* **273**: 7787-7790.

Talanian R.V., Quinlan C., Trautz S., Hackett M.C., Mankovich J.A., Banach D., Ghayur T., Brady K.D. and Wong W.W. (1997) Substrate specificities of caspase family proteases. *J. Biol. Chem.* **272**: 9677-9682.

Tamm I., Wang Y., Sausville E., Scudiero D.A., Vigna N., Oltersdorf T. and Reed J.C. (1998) IAP-family protein Survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases and anticancer drugs. *Cancer Res.* **58**: 5315-5320.

Teitz T., Wei T., Valentine M.B., Vanin E.F., Grenet J., Valentine V.A., Behm F.G., Look T., Lahti J.M. and Kidd V.J. (2000) Caspase-8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. *Nature Med.* **6**: 529-535.

Tewari M., Beidler D.R. and Dixit V.M. (1995a) CrmA-inhibitable cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein during Fas- and tumor necrosis factor-induced apoptosis. *J. Biol. Chem.* **270**: 18738-18741.

Tewari M. and Dixit V.M. (1995) Fas- and Tumour Necrosis Factor-induced apoptosis is inhibited by the poxvirus *crmA* gene product. *J. Biol. Chem.* **270**: 3255-3260.

Tewari M., Telford W.G., Miller R.A. and Dixit V.M. (1995b) CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocyte-mediated apoptosis. *J. Biol. Chem.* **270**: 22705-22708.

Tewari M., Quan L.T., O'Rourke K., Desnoyers S., Zeng Z., Beidler D.R., Poirier G.G., Salvesen G.S. and Dixit V.M. (1995c) Yama/CPP32 beta, a mammalian homologue of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly (ADP-ribose) polymerase. *Cell.* **81**: 801-809.

Thomas H.E., Stunnenberg H.G. and Stewart A.F. (1993) Heterodimerisation of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. *Nature.* **362**: 471-475.

Thome M., Schneider P., Hoffmann K., Fickenscher H., Meinl E., Neipel F., Mattmann C., Burns K., Bodmer J-L., Schroter M., Scaffidi C., Krammer P.H., Peter M.E. and Tschopp J. (1997) Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature.* **386**: 517-521.

Thome M., Hofmannc K., Burns K., Martinon F., Bodmer J.L., Mattmann C. and Tschopp J. (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.* **8**: 885-888.

Thompson C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science.* 267: 1456-1462.

Thornberry N.A., Bull H.G., Calaycay J.R., Chapman K.T., Howard A.D., Kostura M.J., Miller D.K., Molineaux S.M., Weidner J.R., Aunins J., Elliston K.O., Ayala J.M., Casano F.J., Chin J., Ding G.J-F., Egger L.A., Gaffney E.P., Limjuco G., Palyha O.C., Raju S.M., Rolando A.M., Salley J.P., Yamin T-T., Lee T.D., Shively J.E., MacCross M., Mumford R.A., Schmidt J.A. and Tocci M.J. (1992) A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature*. **356**: 768-774.

Thornberry N.A., Rano T.A., Peterson E.P., Rasper D.M., Timkey T., Garcia-Calvo M., Houtzager V.M., Nordstrom P.A., Roy S., Vaillancourt J.P., Chapman K.T. and Nicholson D.W. (1997) A combinatorial appraoch defines specificities of members of the caspase family and granzyme B. *J. Biol. Chem*. **270**: 17907-17911.

Thress K., Henzel W., Shillinglaw W. and Kornbluth S. (1998) Scythe: a novel reaper-binding apoptotic regulator. *EMBO J.* **17**: 6135-6143.

Thress K., Evans E.K and Kornbluth S. (1999) Reaper-induced dissociation of a Scythe-sequestered cytochrome *c*-releasing activity. *EMBO J.* **18**: 5486-5493.

Thummel C.S. (1996) Flies on steroids- *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Gen*. **12**: 307-310.

Troy C.M., Stefanis L., Greene L.A. and Shelanski M.L. (1997) Nedd2 is required for apoptosis after trophic factor withdrawal, but not superoxide dismutase (SOD1) downregulation, in sympathetic neurons and PC12 cells. *J. Neurosci*. **17**: 1911-1918.

Truman J.W. (1984) Cell death in invertebrate nervous systems. *Annu. Rev. Neurosci*. **7**: 171-188.

Tse E. and Rabbitts T.H. (2000) Intracellular antibody-caspase-mediated cell killing: an approach for application in cancer therapy. *Proc. Natl. Acad. Sci. USA*. **97**: 12266-12271.

Tsujimoto Y., Yunis J., Onorato-Showe I., Erikson J., Nowell P.C. and Croce C.M. (1984) Molecular cloning of the chromosomal breakpoint of B-cell lymphomas and leukaemias with the t(11;14) chromosome translocation. *Science*. **224**: 1403-1406.

Tsujimoto Y., Cossman J., Jaffe E. and Croce C.M. (1985) Involvement of the human *bcl-2* gene in human follicular lymphoma. *Science*. **228**: 1440-1443.

Tsujimoto Y. and Croce C.M. (1986) Analysis of the structure, transcripts, and protein products of *bcl-2*, the gene involved in human follicular lymphoma. *Proc. Natl. Acad. Sci. USA*. **83**: 5214-5218.

Tsujimoto Y. (1989) Stress-resistance conferred by high level of *bcl-2*α protein in human B lymphoblastoid cell. *Oncogene*. **4**: 1331-1336.

Uren A.G., Pakusch M., Hawkins C.J., Puls K.L. and Vaux D.L. (1996) Cloning and expression of apoptosis inhibitory protein homologues that function to inhibit apoptosis and/or bind TRAFs. *Proc. Natl. Acad. Sci. USA*. **93**: 4974-4978.

Van Criekinge W., Beyaert R., Van de Craen M., Vandenabeele P., Schotte P., De Valck D. and Fiers W. (1996) Functional characterisation of the pro-domain of interleukin-1β-converting enzyme. *J Biol. Chem*. **271**: 27245-27248.

Varfolomeev E.E., Schuchmann M., Luria V., Chiannilkulchai N., Beckmann J.S., Mett I.L., Rebrikov D., Brodianski V.M., Kemper O.C., Kollet O., Lapidot T., Soffer D., Sobe T., Avraham K.B., Goncharov T., Holtmann H., Lonai P. and Wallach D. (1998) Targeted disruption of the mouse caspase-8 gene ablates cell death induction by the TNF receptors., Fas/Apo1 and DR3 and is lethal prenatally. *Immunity*. **9**: 267-276.

Varkey J., Chen P., Jemmerson R. and Abrams J.M. (1999) Altered cytochrome c display precedes apoptotic cell death in *Drosophila*. *J. Cell Biol*. **144**: 701-710.

Vaux D.L., Aguila H.L. and Weissman I.L. (1992a) Bcl-2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell mediated killing. *Int. Immunol.* **4**: 821-824.

Vaux D.L., Weissman I.L. and Kim S.K. (1992b) Prevention of programmed cell death in *Caenorhabditis elegans* by human *bcl-2*. *Science*. **258**: 1955-1957.

Vaux D.L., Haecker G. and Strasser A. (1994) An evolutionary perspective on apoptosis. *Cell.* 76: 777-779.

Vaux D.L. and Korsmeyer S.J. (1999) Cell death in development. *Cell.* **96**: 245-254.

Veis D.J., Sorenson CM., Shutter JR. and Korsmeyer SJ. (1994) *Bcl-2*-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell.* **75**: 229-240.

Verhagen A.M., Ekert P.G., Pakusch M., Silke J., Connolly L.M., Reid G.E., Moritz R.L., Simpson R.J. and Vaux D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell.* **102**: 43-53.

Vernooy S.Y., Copeland J., Ghaboosi N., Griffin E.E., Yoo S.J. and Hay B.A. (2000) Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol.* **150**: F69-F75.

Vucic D., Kaiser W.J., Harvey A.J. and Miller L.K. (1997) Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc. Natl. Acad. Sci. USA.* **94**: 10183-10188.

Vucic D., Kaiser W.J. and Miller L.K. (1998) Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. *Mol. Cell. Biol.* **18**: 3300-3309.

Walker N.P.C., Talanian R.V., Brady K.D., Dang L.C., Bump N.J., Ferenz C.R., Franklin S., Ghayur T., Hackett C.R., Hammill L.D., Herzog L., Hugunin M., Houy W., Mankovich J.A., McGuiness L., Orlewicz E., Paskind M., Pratt C.A., Reis P., Summani A., Terranova M., Welch J.P., Xiong L., Müller A., Tracey D.E., Kamen R. and Wong W.W. (1994) Crystal structure of the cysteine protease interleukin-1β converting enzyme: a (p20/p10)2 homodimer. *Cell.* **78**: 343-352.

Wallach D. (1997) Placing death under control. *Nature.* **388**: 123-126.

Wang K., Yin X-M., Chao D.T., Milliman C.L. and Korsmeyer S.J. (1996a) BID: a novel BH3 domain-only death agonist. *Genes Dev.* **10**: 2859-2869.

Wang L., Miura M., Bergeron L., Zhu H. and Yuan J. (1994) *Ich-1* an *Ice/ced-3*-related gene, encodes both positive and negative regulators of programmed cell death. *Cell.* **78**: 739-750.

Wang X., Pai J., Wiedenfeld E.A., Medina J.C., Slaughter C.A., Goldstein J.L. and Brown M.S. (1995) Purification of an interleukin-1β converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. *J. Biol. Chem.* **270**: 18044-18050.

Wang X., Zelenski N.G., Yang G., Sakai J., Brown M.S. and Goldstein J.L. (1996b) Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. *EMBO J.* **15**: 1012-1020.

Wang S.L., Hawkins C.J., Yoo S.J., Muller H.A. and Hay B.A. (1999) The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell.* 98: 453-463.

Warrick J.M., Paulson H.L., Gray-Board G.L., Bui Q.T., Fischbeck K.H., Pittman R.N. and Bonini N.M. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell*. **93**: 939-949.

Watters D. and Waterhouse N. (1998) Proteolytic targets in cell death. In Kumar S. (Ed) Apoptosis: Mechanisms and role in disease. Vol. **24**: 25-44.

Weinmaster G. (2000) Notch signal transduction: a real Rip and more. *Curr. Opin. Gen. Dev.* **10**: 363-369.

White K., Grether M.E., Abrams J.M., Young L., Farrell K. and Steller H. (1994) Genetic control of programmed cell death in *Drosophila*. *Science*. **264**: 677-683.

White K., Tahaoglu E. and Steller H. (1996) Cell killing by the *Drosophila* gene *reaper*. *Science*. **271**: 805-807.

White K. (1999) The third horseman takes wing. *Nat. Cell Biol.* 1: E123-E124.

Wilson K.P., Black J-A.F., Thomson J.A., Kim E.E., Griffith J.P., Navia M.A., Murcko M.A., Chambers S.P., Aldape R.A., Raybuck S.A. and Livingston D.J. (1994) Structure and mechanism of interleukin-1β converting enzyme. *Nature*. **370**: 270-275.

Wing J.P. Zhou L. Shwartz Lawrence M. and Nambu J.R. (1998) Distinct cell killing properties of the *Drosophila reaper*, *head involution defective* and *grim* genes. *Cell Death Differ*. **5**: 930-939.

Wolff T. and Ready D.F. (1991) Cell death in normal and rough eye mutants of *Drosophila*. *Development*. **113**: 825-839.

Wu D., Wallen H.D. and Nunez G. (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science.* **275**: 1126-1129.

Wu Y-C. and Horvitz H.R. (1998a) *C. elegans* phagocytosis and cell migration protein CED-5 is similar to human DOCK180. *Nature.* **392**: 501-509.

Wu Y-C. and Horvitz H.R. (1998b) The *C. elegans* cell corpse engulfment gene *ced-7* encodes a protein similar to ABC transporters. *Cell.* **93**: 951-960.

Wyllie A.H., Kerr J.F.R. and Currie A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68**: 251-306.

Xue D. and Horvitz H.R. (1995) Inhibition of the *Caenorhabditis elegans* cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature.* **377**: 248-251.

Xue D., Shaham S. and Horvitz H.R. (1996) The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of human CPP32 protease. *Genes and Dev.* **10**: 1073-1083.

Yang E., Zha J., Jockel J., Boise L.H., Thompson C.B. and Korsmeyer S.J. (1995) Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces Bax and promotes cell death. *Cell.* **80**: 285-291.

Yang X., Chang H.Y. and Baltimore D. (1998) Autoproteolytic activation of procaspases by oligomerization. *Mol. Cell.* **1**: 319-325.

Yang Y., Fang S., Jensen J.P., Weissman A.M. and Ashwell J.D. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteosomes in response to apoptotic stimuli. *Science.* **288**: 874-877.

Yokoyama H., Mukae N., Sakahira H., Okawa K., Iwamatsu A. and Nagata S. (2000) A novel activation mechanism of caspase-activated DNase from *Drosophila melanogaster*. *J. Biol. Chem.* **275**: 12978-12986.

Yoshida H., Kong Y-Y., Yoshida R., Elia A.J., Hakem A., Hakem R., Penninger J.M. and Mak T. (1998) Apaf-1 is required for mitochondrial pathways of apotosis and brain development. *Cell.* **94**: 739-750.

Yuan J. and Horvitz H.R. (1990) Genetic mosaic analyses of *ced-3* and *ced-4*, two genes that control programmed cell death in the nematode *C. elegans*. *Dev. Biol.* **138**: 33-41.

Yuan J. and Horvitz H.R. (1992) The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development.* **116**: 309-320.

Yuan J., Shaham S., Ledoux S., Ellis H. and Horvitz H.R. (1993) The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell.* **75**: 641-652.

Zhang H., Huang Q., Ke N., Matsuyama S., Hammock B., Godzik A. and Reed J.C (2000) *Drosophila* pro-apoptotic Bcl2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J. Biol. Chem.* **275**: 27303-27306.

Zheng T.S., Hunot S., Kuida K. and Flavell R.A. (1999) Caspase knockouts: matters of life and death. *Cell Death Differ.* **6**: 1043-1053.

Zheng T.S., Hunot S., Kuida K., Momoi T., Srinivasan A., Nicholson D.W., Lazebnik Y. and Flavell R.A. (2000) Deficiencies in caspase-9 or caspase-3 induces compensatory caspase activation. *Nature Med.* **6**: 1241-1247.

Zhou L., Scnitzler A., Agapite J., Schwartz L.M., Stellar H. and Nambu J.R. (1997) Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl. Acad. Sci. USA*. **94**: 5131-5136.

Zhou L., Zhiwei S., Tittel J. and Stellar H. (1999) HAC-1, a *Drosophila* homolog of Apaf-1 and CED-4, functions in developmental and radiation-induced apoptosis. *Mol. Cell.* **4**: 745-755.

Zhou Q., Snipas S., Orth K., Muzio M., Dixit V.M. and Salvasen GS. (1997) Target protease specificity of the viral serpin CrmA; analysis of five caspases. *J. Biol. Chem.* **272**: 7797-7800.

Zou H., Henzel W.J., Liu X., Lutschg A. and Wang X. (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*. **90**: 405-413.

Zou H., Li Y., Liu X. and Wang X. (1999) An APAF-1/cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem*. **274**: 11549-11556.

Thesis Amendments

Chapter 2

Page 51 — Missing primer sequence used for the amplification of full length DECAY.
DEC_{FL}: 5'-*CCCAAGCTTGCC*ATGGACGACACCGACTTC-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:
Brand A.H. and Perrimon N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* **118**: 401-415.