An analysis of DRONC function and its regulation of expression during *Drosophila* development

By

Tasman James Daish, B.Sc (Hons)

Enrolled through the Department of Medicine,

Faculty of Health Science, The University of Adelaide

Research conducted at the Institute of Medical and Veterinary Science,

Department of Haematology, Hanson Institute, Adelaide

A thesis submitted for the degree of Doctor of Philosophy,

In the Faculty of Health Science, The University of Adelaide

December 2004

Table of Contents

Abstract

Correct development of multicellular organisms requires the programmed removal of supernumerary, redundant, or damaged cells, a process achieved by apoptosis. Apoptosis, or Programmed Cell Death (PCD) is executed by caspases, a highly conserved family of cysteine proteases. The removal of redundant larval tissues during metamorphosis is controlled by the steroid hormone ecdysone. Ecdysone signalling is mediated by the nuclear receptor heterodimer EcR/Usp, which in turn transcriptionally activates a host of transcription factors which then go on to regulate genes essential for PCD, like caspases. The apical caspase *dronc* is upregulated in the larval midgut and salivary glands prior to their destruction and is dependent on the BR-C and E93 transcription factors. To further understand the role of *dronc* in development, a *dronc* mutant fly was generated and a transgenic promoter-reporter strategy was employed to investigate *dronc* regulation.

Larval organs from *dronc* mutants lack dying cells and, when irradiated, fail to show a radiation-induced PCD response. The midguts from *dronc* mutants undergo apoptosis and have high caspase activity. These data indicate that a *dronc*-independent caspase activation pathway is active in the midgut. Salivary glands from *dronc* mutants failed to be removed and have reduced caspase activity. Consequently it is clear that the role of DRONC differs significantly between the midgut and salivary glands.

The employment of a transgenic *dronc* promoter-*LacZ* reporter system identified promoter regions essential for the correct temporal and spatial expression of *dronc*. A region of the *dronc* promoter between 1.1kb and 2.8kb has elements essential for *LacZ* expression in salivary glands. This region was also dependent on the BR-C and E93

transcription factors for salivary gland expression. A functional ecdysone receptor binding element (EcRBE) was identified in the *dronc* proximal promoter. The EcR-B1 isoform directly binds this EcRBE and is necessary for correct *dronc* expression in the larval salivary glands.

This work revealed some novel findings regarding the role of DRONC in development and the availability of a specific *dronc* mutant now makes it possible to explore some of the recently published non-apoptotic roles of *dronc*. This work aids in understanding how nuclear hormones control transcription and shows *dronc* to be an ideal model gene to explore these molecular and genetic processes.

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 my consent for this thesis to be made available for loan and photocopying.

Acknowledgements

Firstly I must thank Professor Sharad Kumar who gave me the opportunity to work and study in his laboratory and under whose supervision I have endeavoured to complete my PhD studies. Without his example and efforts I would have neither published nor completed these studies. I am also very grateful for the persistence and support from all members of the lab, past and present, who helped me get through the tough times (Loretta, Stuart, Belinda, Linda and Andrew). Thanks to Dr Dimitrios Cakouros for practical guidance toward the direction and formulation of many parts of my research, and whom is responsible for all EMSA analyses and *l(2)mbn* cell line experiments in chapter 5. Also thankyou to Kathryn Mills who has participated in many aspects of the *dronc* mutant work, including the P-element excision, complementation construct cloning, acridine orange staining, genetic interaction and irradiation experiments.

Thanks must go to Dr Eric Baehrecke, Dr John Abrams and Dr Tetyana Shandala for scientific and methodological advice. Also I must acknowledge Drs Leonie Quinn and Helena Richardson without whose guidance and support I could not have progressed in science.

When in doubt I always depended on the rock solid support and encouragement of Maura and family. My beautiful daughters, Ashley and Harriet, came along to remind me that life is love and family and even when in despair I was still awash with good fortune.

Publications

Daish, T. J., Mills, K., and Kumar, S. (2004). *Drosophila* Caspase DRONC Is Required for Specific Developmental Cell Death Pathways and Stress-Induced Apoptosis. Dev Cell *7*, 909-915.

Chew, S. K., Akdemir, F., Chen, P., Lu, W. J., Mills, K., **Daish, T.J.,** Kumar, S., Rodriguez, A., and Abrams, J. M. (2004). The Apical Caspase *dronc* Governs Programmed and Unprogrammed Cell Death in *Drosophila*. Dev Cell *7*, 897-907.

Cakouros, D., **Daish, T. J**., and Kumar, S. (2004a). Ecdysone receptor directly binds the promoter of the *Drosophila* caspase *dronc*, regulating its expression in specific tissues. J Cell Biol *165*, 631-640.

Cakouros, D., **Daish, T. J**., Mills, K., and Kumar, S. (2004b). An arginine-histone methyl transferase, CARMER, coordinates ecdysone-mediated apoptosis in *Drosophila* cells. J Biol Chem *279*, 18467-18471.

Quinn, L., Coombe, M., Mills, K., **Daish, T.J**., Colussi, P., Kumar, S., and Richardson, H. (2003). Buffy, a *Drosophila* Bcl-2 protein, has anti-apoptotic and cell cycle inhibitory functions. Embo J *22*, 3568-3579.

Daish, T. J., Cakouros, D., and Kumar, S. (2003). Distinct promoter regions regulate spatial and temporal expression of the *Drosophila* caspase *dronc*. Cell Death Differ *10*, 1348-1356.

Cakouros, D., **Daish, T.J**., Martin, D., Baehrecke, E. H., and Kumar, S. (2002). Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in Drosophila is regulated by Broad-Complex. J Cell Biol *157*, 985-995.

Harvey, N. L., **Daish, T.J**., 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 *276*, 25342-25350.

Chapter 1

Introduction

1.1 Programmed cell death (PCD)

Programmed cell death (PCD) is required for the normal development of all multicellular organisms. Removal of damaged, redundant, or superfluous cells is essential for correct body and tissue patterning (Baehrecke, 2002; Jacobson et al., 1997). Only recently has PCD been recognised as an important developmental process which, when faulty or deregulated, contributes toward the manifestation of a host of disease states including various cancers, immune and neurodegenerative disorders (Bickler and Donohoe, 2002; Fearnhead, 2004; Thompson, 1995; Vaux and Flavell, 2000; Waldmeier and Tatton, 2004; Yuan and Yankner, 2000). Consequently the study of PCD will ultimately aid in the better understanding of human disease and from this, therapies to prevent or alleviate such conditions can be developed.

The most common form of PCD is apoptosis. Apoptosis is typically characterised by DNA fragmentation, nuclear contraction, membrane blebbing and condensation of the cytosol (Kerr et al., 1972; Wyllie et al., 1980). Subsequent removal of apoptotic cells is achieved by macrophage engulfment thereby avoiding an inflammatory response in the surrounding healthy tissue (Kerr et al., 1972). Apoptosis requires the activation of a conserved class of cysteine proteases called caspases to effect destruction of the cell or tissue (Lee and Baehrecke, 2001). Consequently caspases can be studied as potential candidates for therapeutic targets to modify or prevent aberrant PCD.

1.2 Conservation of the cell death machinery

1.2.1 *Caenorhabditis elegans*

Discovery of an evolutionarily conserved programmed cell death pathway involved the identification of three essential genes in the nematode *Caenorhabditis*

elegans that are required for the correct removal of a discrete population of 131 cells during development (Sulston and Horvitz, 1977). These genes, *egl-1, ced-4* and *ced-3* act co-ordinately to ensure that the same set of cells die (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner et al., 1992). A fourth gene, *ced-9* functions to protect the remaining 959 cells from death (Ellis and Horvitz, 1986).

CED-3 is the caspase representative in the worm, is autocatalytically processed following an aspartate residue, and functions as the primary effector of cell death (Yuan et al., 1993). CED-4 is an adaptor molecule that functions to effect cell death by binding to and activating CED-3 (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Seshagiri and Miller, 1997; Wu et al., 1997a). The survival function of CED-9 comes from its ability to interact with CED-4 and prevent it activating CED-3 (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Spector et al., 1997; Wu et al., 1997a; Wu et al., 1997b). EGL-1 interacts with CED-9 to prevent it from sequestering CED-4, thereby allowing CED-3 activation and cell death (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Conradt and Horvitz, 1998; Shaham and Horvitz, 1996). As described below, the worm death genes represent gene families in more complex animals.

1.2.2 Mammals

Caspase activation signals the commitment of a cell to die and leads to the cleavage of multiple cellular protein targets. Caspases are so termed because they are cysteinyl aspartate-specific proteinases that cleave their target proteins after an aspartate residue (Margolin et al., 1997). In normal or healthy cells, caspases exist as inactive zymogens that become proteolytically cleaved and activated upon receipt of a death signal (Salvesen and Abrams, 2004). In general, active caspases are

composed of two sets of dimers consisting of a large and small caspase subunit (Stennicke and Salvesen, 1999).

Caspases are categorised into two classes based on the length of their N-terminal prodomains. Class I or initiator caspases have a long prodomain that contains protein-protein interaction motifs involved in caspase activation and regulation (figure 1.1) (Kumar and Colussi, 1999; Nicholson, 1999; Riedl and Shi, 2004). The class II or effector caspases are in turn activated by the activated initiator caspases and these go on to cleave numerous protein targets (Kumar and Colussi, 1999; Nicholson, 1999; Salvesen and Abrams, 2004; Shi, 2002; Thornberry and Lazebnik, 1998).

In mammals, as in the worm, regulation of caspase activation occurs through interaction with anti- and pro-death molecules leading to oligomerisation and cleavage of the caspase subunits. Figure 1.2 shows a schematic diagram of the conserved caspase activation pathways leading to cell death in *C. elegans*, mammals, and *Drosophila melanogaster* (Bratton and Cohen, 2003; Degterev et al., 2003). The two major mammalian death pathways are termed the ‘extrinsic’ or receptor-mediated cell death pathway and the intrinsic or ‘mitochondrial’ death pathway. Both pathways eventually lead to the activation of effector caspases.

The extrinsic pathway involves formation of a death-inducing signalling complex (DISC) that leads to caspase-8 and then effector caspase activation (Peter and Krammer, 2003; Li et al., 1998). DISC formation results from Fas ligand binding to the Fas receptor, then recruitment of the adaptor molecule FADD, which in turn recruits pro-caspase-8, triggering its proximity-induced autoactivation (Donepudi et al., 2003). Activated caspase-8 functions also in the intrinsic pathway through

interaction with the BH3-only protein BID, which results in cytochrome c release from the mitochondria (Wei et al., 2000).

Multiple death triggers can activate the intrinsic death pathway resulting in the release from the mitochondria of pro-death molecules like cytochrome c and Smac/Diablo due to loss of mitochondrial membrane permeability (figure 1.2) (van Gurp et al., 2003). Cytochrome c release results in formation of the apoptosome, a large pro-death complex composed of cytochrome c, the adaptor molecule Apaf-1 and caspase-9. Formation of the apoptosome results in caspase-9 activation and subsequent activation of the effector caspase, caspase-3. The balance between activation and suppression of the death program is modulated in part by blocking the inhibitor of apoptosis (IAP) proteins by the mitochondrial-released Smac/Diablo protein (Chai et al., 2000). This occurs via interactions similar to that occurring between the *Drosophila* RHG proteins and DIAP1 (Liu et al., 2000; Wu et al., 2000).

A critical group of molecules regulating the intrinsic pathway are the Bcl-2 family proteins. Bcl-2 is the functional homologue of the anti-apoptotic CED-9 (Hengartner and Horvitz, 1994). Bcl-2 family members, categorised on the presence or absence of Bcl-2 homology domains (BH1-4), have distinct pro- and antiapoptotic roles. The BH domains are involved in protein-protein interactions between the pro- and antiapoptotic Bcl-2 family members to modulate their respective roles. Murine prosurvival *Bcl-2* and *Bcl-X_L* mutants have discrete lethal phenotypes (Motoyama et al., 1995; Veis et al., 1993). In contrast, the proapoptotic Bcl-2 family proteins (eg. Bax, Bak and Bok), are defined by the absence of a BH4 domain and have a degree of functional redundancy (Knudson et al., 1995; Lindsten et al., 2000). The balance between these molecules decides the release of cytochrome-c and Smac/Diablo from the mitochondria. Another group of Bcl-2 like molecules, the EGL-1 homologues, are

Figure 1.1 Caspase protein domain structure across species.

All caspases are composed of a large and small subunit of variable size, with the initiator or upstream members having an N-terminal prodomain containing death effector domains (DED) or caspase recruitment domains (CARD), both required for protein-protein interactions. The catalytic Cysteine residue is indicated by a vertical white line. Modified from Riedl and Shi (2004).

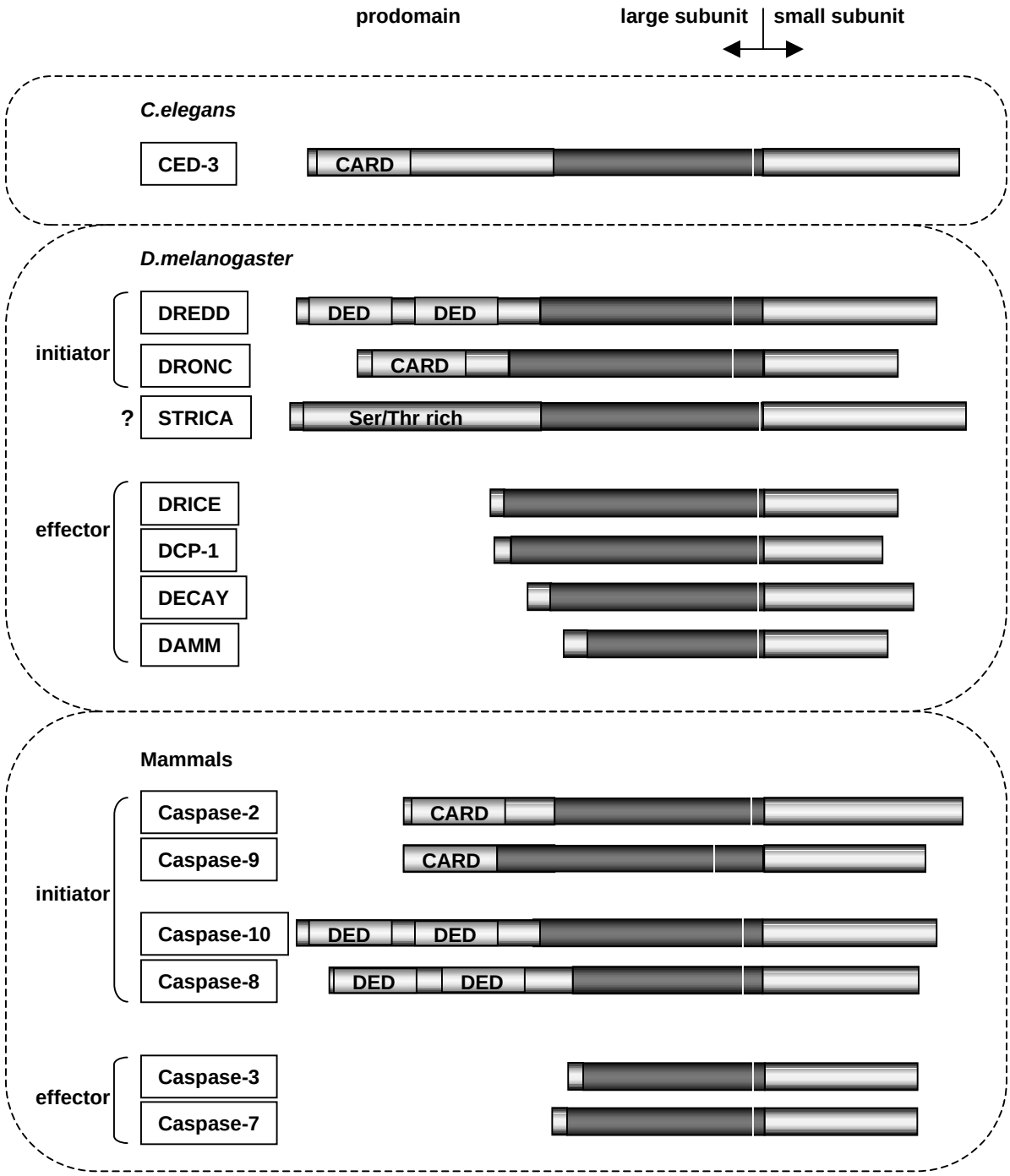
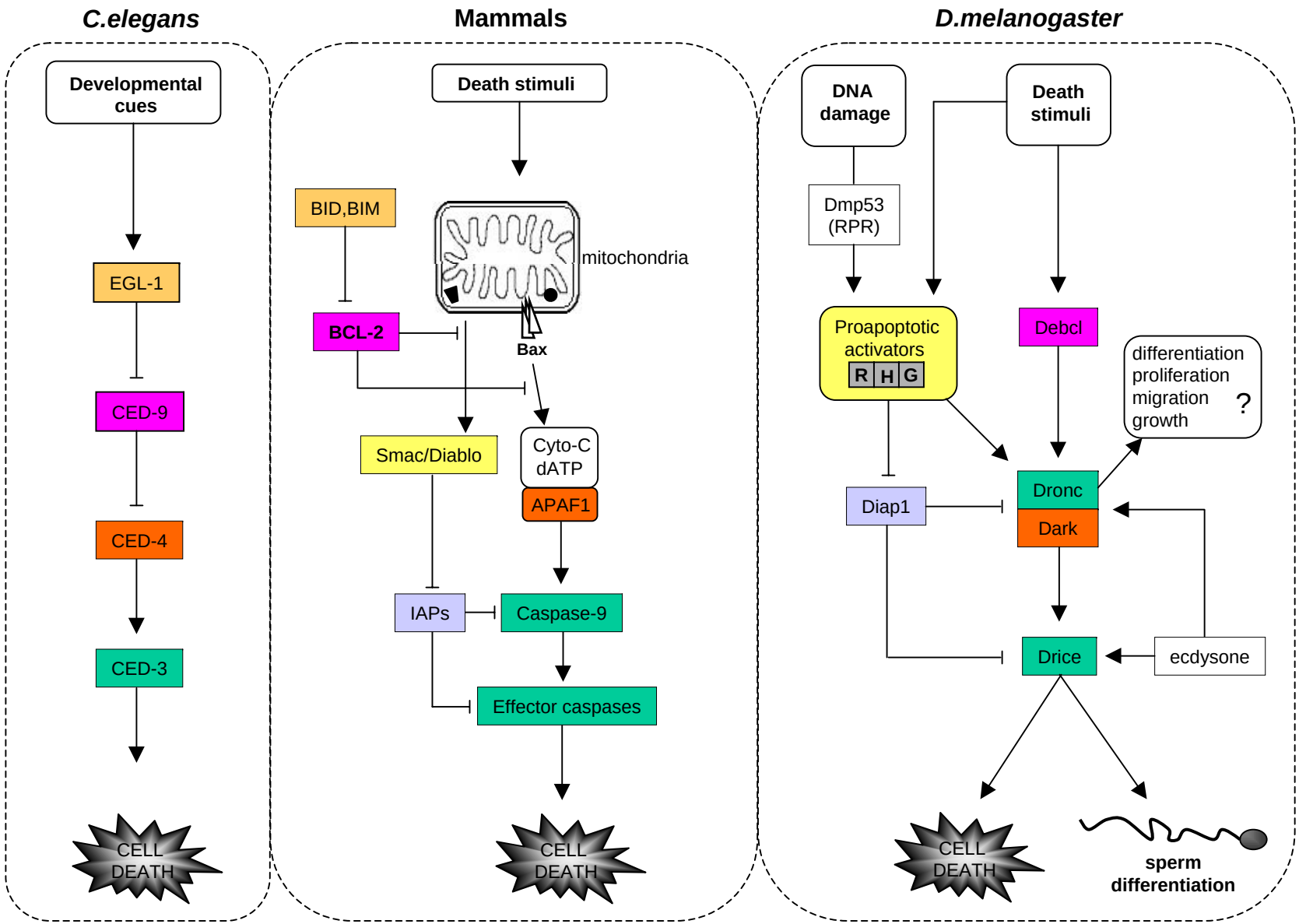

prodomain
large subunit
small subunit
C.elegans
CED-3
CARD
D.melanogaster
initiator
DREDD
DED
DED
DRONC
CARD
?
STRICA
Ser/Thr rich
effector
DRICE
DCP-1
DECAY
DAMM
Mammals
initiator
Caspase-2
CARD
Caspase-9
CARD
Caspase-10
DED
DED
Caspase-8
DED
DED
effector
Caspase-3
Caspase-7

Figure 1.2 Conservation of the cell death pathways.

Left panel; cell death machinery in *C. elegans*. Four key genes code for the proteins regulating cell death and survival in the nematode. The adaptor molecule CED-4 binds to and activates CED-3 through a CARD-CARD interaction. The BH3-only protein, EGL-1, when activated by death signalling molecules, sequesters the Bcl-2 homologue CED-9 from CED-4 which allows CED-3 activation and cell death. Middle panel; the 'intrinsic' cell death machinery in mammals. Release of Smac/Diablo and cytochrome c from mitochondria allows inhibition of IAPs and formation of the apoptosome (cytochrome c/ dATP/ APAF-1/ caspase-9), both leading to the activation of caspase-9. Activity of proapoptotic Bcl-2 homologues cause Bcl-2 inhibition and the formation of channels through the mitochondrial membrane permitting Smac/Diablo and Cytochrome c release. Right panel; the cell death machinery in *D. melanogaster.* Death stimuli cause the RHG death activator proteins to inhibit the IAPs allowing DRONC and DRICE activation. Activation of DRONC occurs through interaction with the adaptor molecule DARK, and involves the RHG proteins, the hormone ecdysone, and the Bcl-2-like protein, Debcl. Recent evidence shows DRONC may be involved in diverse non-apoptotic processes. Modified from Riedl and Shi (2004).


C.elegans
Developmental cues
EGL-1
CED-9
CED-4
CED-3
CELL DEATH
Mammals
Death stimuli
BID,BIM
mitochondria
BCL-2
Bax
Smac/Diablo
Cyto-C
dATP
APAF1
IAPs
Caspase-9
Effector caspases
CELL DEATH
D.melanogaster
DNA damage
Death stimuli
Dmp53 (RPR)
Proapoptotic activators
R H G
Debcl
differentiation
proliferation
migration
growth
?
Diap1
Dronc
Dark
Drice
ecdysone
CELL DEATH
sperm differentiation

the proapoptotic BH3-only proteins Bid, Bim, Bad, Puma and Noxa (Puthalakath and Strasser, 2002). The BH3-only proteins have been shown to function through the proapoptotic Bcl-2 proteins Bax and Bak (Cheng et al., 2001; Wei et al., 2001).

1.2.3 *Drosophila melanogaster*

1.2.3.1 A model system to study cell death

Drosophila melanogaster has long been recognised as a valuable experimental tool when investigating fundamental biological processes. This is in part due to their short life cycle and convenient size, making developmental or hereditary studies and containment an amenable and affordable exercise. *Drosophila* have a life cycle from egg deposition to adulthood of approximately 11 days at 25^{o}C, comprising well defined and phenotypically identifiable developmental transition boundaries from the larval to the prepupal and pupal stages (figure 1.3). The embryology and metamorphosis of *Drosophila* has been comprehensibly documented over the last century allowing detailed comparative developmental studies. The value of this organism as a genetic tool was enhanced recently by the sequencing of the genome along with the establishment of P-element libraries making mutant analysis or generation through imprecise excision strategies a much easier task. The yeast GAL4; UAS and the FLP/FRT system presents *Drosophila* as an ideal system for the molecular geneticist (Duffy, 2002). PCD pathway components are also conserved in the fly (figure 1.2). The fact that PCD genes are highly conserved throughout evolution makes *Drosophila* an ideal experimental system to shed light on the more complex PCD machinery existing in higher order organisms.

PCD is an essential mechanism for correct embryogenesis to occur, commencing around seven hours (stage 11) following egg deposition and continuing

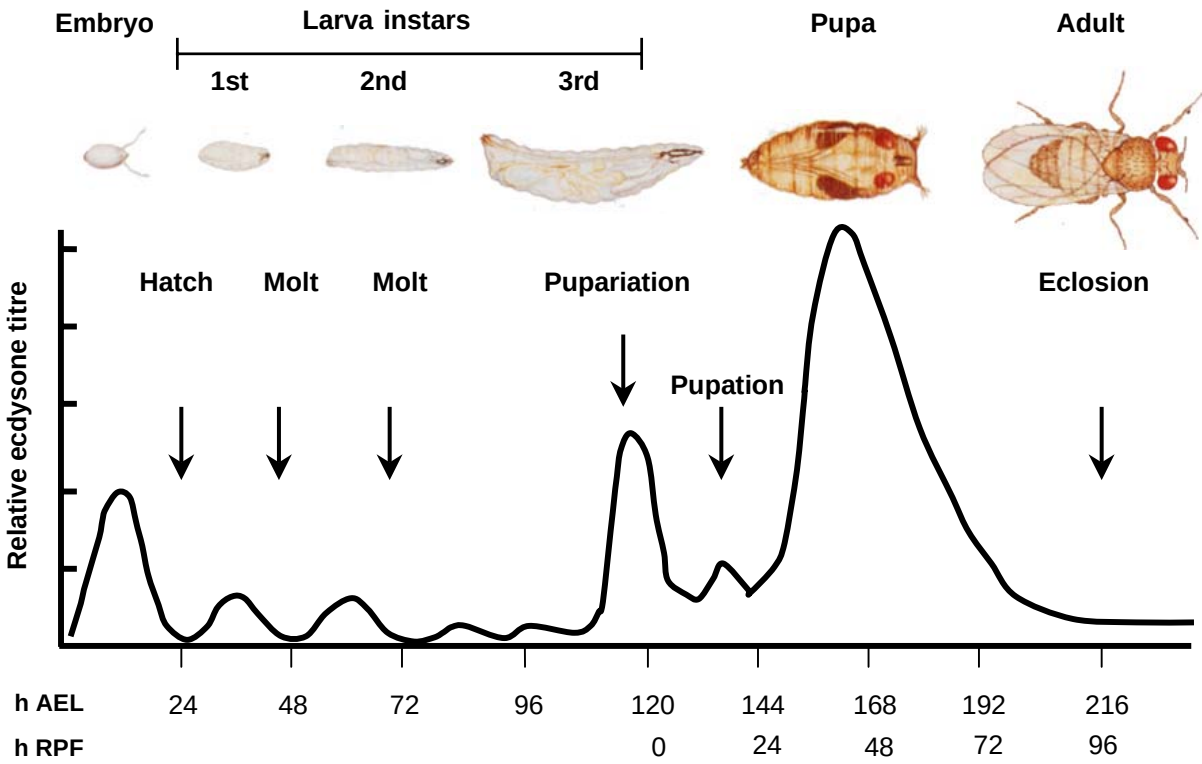


Figure 1.3 Ecdysone titre throughout *Drosophila* development.

Increases in the ecdysone titre punctuate each developmental stage of the *Drosophila* life cycle marking the transitions from embryo into the larval stage, progression of the two larval moults, and pupariation of the third instar larva, metamorphosis, and eclosion of the adult fly. At 25°C, development from egg deposition to eclosion takes around 11 days. A small ecdysone pulse around 10 hours after puparium formation causes head eversion and entry into the pupal stage proper. Developmental times are displayed as hours after egg laying (h AEL) and hours relative to puparium formation (h RPF). Adapted from Riddiford LM (1993).

during segmentation to control cell fate specifications along with central nervous system modifications organising axonal networking (Abrams et al., 1993). Abrams et al. (1993) described typically vertebrate hallmarks of apoptosis in Acridine Orange (AO) positive embryonic cells, namely nuclear condensation and fragmentation followed by macrophage engulfment. Furthermore, this cell death was widespread and of a generally reproducible pattern at all embryonic stages indicative of a pre-programmed death schedule. The adult fly requires PCD events to ensure correct retinal patterning and the elimination of the 16 nurse cells in the formation of mature eggs prior to fertilisation and deposition (Buszczak and Cooley, 2000; Rusconi et al., 2000).

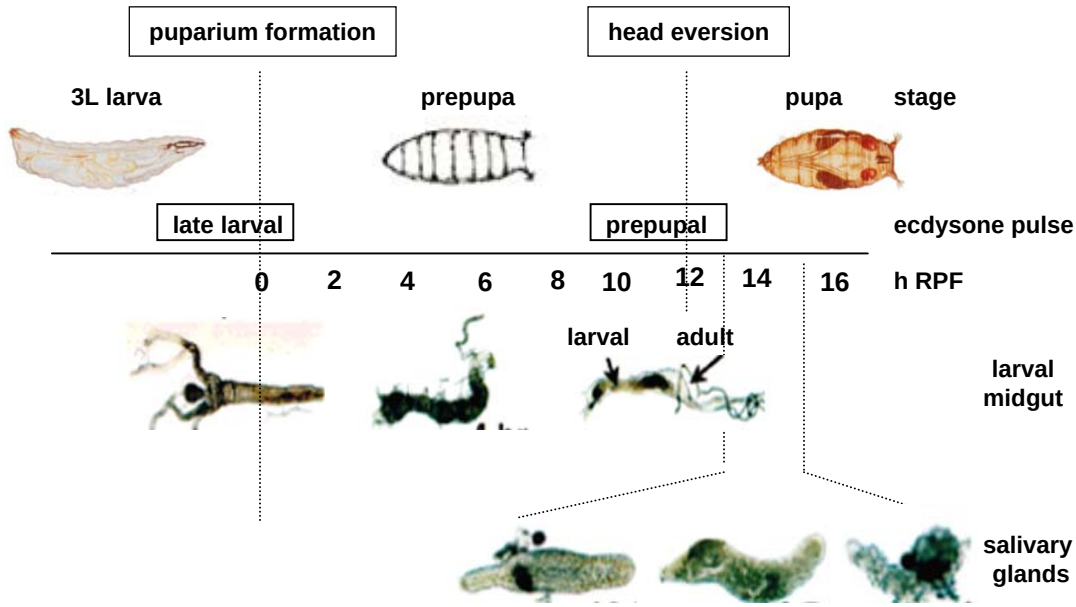
Each developmental transition through the *Drosophila* life cycle is punctuated by increases in the titre of the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) which orchestrates the temporal developmental transitions (figure 1.3) (Riddiford, 1993). The late third larval instar ecdysone peak initiates pupation and entry into the prepupal stage, ending approximately 12 hours later with another ecdysone peak causing head eversion and entry into the pupal stage proper.

The onset of metamorphosis offers a unique model system to study cell death mechanisms as the larval midgut and salivary glands rapidly undergo histolysis and removal during this period (figure 1.4 A). Midgut removal commences at puparium formation while salivary gland removal commences around 14 hours relative to puparium formation (h RPF). These obsolete larval tissues are replaced during the prepupal and pupal period by the proliferation of adult tissues originating from clusters of tissue-specific progenitor cells (Bodenstein, 1965; Robertson, 1936). In particular, the salivary glands have historically been used, notably by Ashburner and colleagues, to investigate how a hormone can induce complex transcriptional cascades by

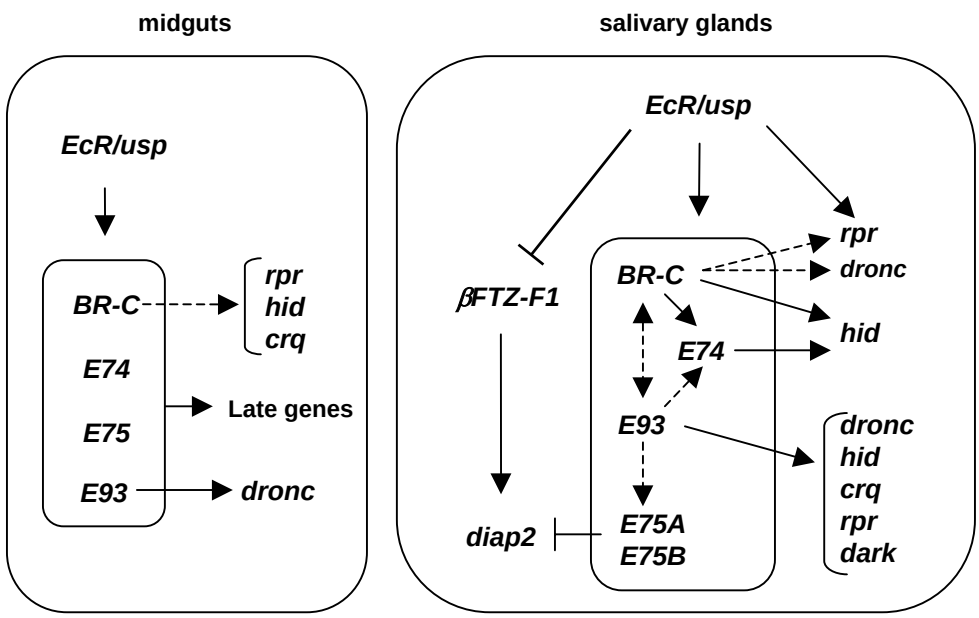
Figure 1.4 Ecdysone regulates the destruction of the larval midguts and salivary glands.

(A) Timing of the destruction of the larval midguts and salivary glands. Transition of the third instar larvae to the prepupae, initiated by the late larval ecdysone pulse, signals the destruction of the larval midgut over the ensuing 10 hours. Midguts show regression of the gastric caecae, proventriculus, and general contraction. The late prepupal ecdysone pulse at 10 hours after puparium formation induces head eversion, destruction of the salivary glands at around 14 hours after puparium formation, and formation of the pupa and adult structures. This figure was modified from Thummel (2001). **(B)** The hormone-induced transcriptional hierarchy in the midgut and salivary glands. The transcriptional dependencies between the transcription factors themselves and the core death genes are indicated with arrows, solid arrows indicating a requirement for transcription, broken lines indicating a requirement for maximal transcription. This figure is modified from data in Baehrecke, E (2000), and Lee et al. (2002a, 2002b).

A


puparium formation
head eversion
3L larva
prepupa
pupa
stage
late larval
prepupal
ecdysone pulse
0
2
4
6
8
10
12
14
16
h RPF
larval
adult
larval midgut
salivary glands

B


midguts
salivary glands
EcR/usp
BR-C
E74
E75
E93
rpr
hid
crq
Late genes
dronc
EcR/usp
βFTZ-F1
BR-C
E74
E93
diap2
E75A
E75B
rpr
dronc
hid
dronc
hid
crq
rpr
dark

visualising sequences of chromosome puffing along the large salivary gland polytene chromosomes during larval and metamorphic development (Ashburner, 1967; Ashburner and Richards, 1976). These investigators observed the emergence of two sets of temporally discrete puffing patterns induced by the late larval ecdysone pulse, the first pattern comprising half a dozen 'early' puffs followed by a much larger set of 'late' puffs (>100). Ashburners' model proposed that ecdysone directly induced the early puff genes, which, as well as repressing their own expression, also directly induced the much larger 'late' set of genes. These late genes were thought to ultimately orchestrate the rapid removal of the glands ~12 hours later following the late prepupal ecdysone pulse. This hierarchical transcriptional cascade, as discussed in detail below, results in the tissue- and stage-specific expression of the core cell death machinery.

1.2.3.2 The *Drosophila* PCD machinery

A significant step toward understanding the cell death pathway and the machinery essential for its execution came with the identification of the *H99* deficiency genes; *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* (Chen et al., 1996; White et al., 1994). These genes are required to mediate the death signal, are transcriptionally upregulated in response to a death signal, and act in synchrony to effect cell death in multiple tissues. These genes induce death when ectopically expressed and coexpression of the baculoviral p35 protein blocks this death indicating the pathway leads to caspase activation (Chen et al., 1996; White et al., 1994).

Although functioning as key activators of cell death in multiple developmental settings, RPR and HID differ from GRIM in that they are ecdysone-responsive and

involved in the removal of the larval midgut and salivary glands at metamorphosis (Yin and Thummel, 2004). The *rpr* promoter is directly transcriptionally activated by p53 through its *Dmp53* response element (*p53RE*) following radiation exposure indicating a role of RPR in the DNA damage response pathway (Brodsky et al., 2000). *hid* is documented as also being expressed in cells which are not destined to die suggesting a non-apoptotic role exists for this cell death activator (Grether et al., 1995).

The mode of action the *H99* proteins use to activate the death pathway resides in their short N-terminal RHG motif (Christich et al., 2002; Wing et al., 1999; Wing et al., 2002). This motif mediates interactions with the *Drosophila* Inhibitor of Apoptosis proteins (DIAP1 and DIAP2) allowing caspase activation and cell death while directing these sequestered proteins to the ubiquitination pathway (Martin, 2002).

IAPs block caspase activity by physical binding through two amino-terminal baculoviral inhibitor of apoptosis repeat (BIR) motifs (Martin, 2002). The *diap1* mutant *thread* was isolated using an *H99* enhancer screen in the fly eye (Hay et al., 1995). Deregulation of PCD in *thread* animals results from the unchecked processing and activation of DCP-1, DRICE and DRONC.

1.3 *Drosophila* caspases

There are seven caspases in *Drosophila*; *dcp-1*, *dredd*, *drice*, *dronc*, *decay*, *strica* and *damm* (Chen et al., 1998; Dorstyn et al., 1999a; Dorstyn et al., 1999b; Doumanis et al., 2001; Fraser and Evan, 1997; Harvey et al., 2001; Song et al., 1997). These proteins, as in mammals, can be grouped into upstream or initiator caspases and downstream or effector caspases based on the presence or absence of long prodomains (figure 1.1). The initiator caspases *dredd* and *dronc* have long

prodomains containing protein-protein interaction motifs required for adaptor molecule interactions leading to activation. DRONC is the only caspase recruitment domain (CARD) containing caspase in the fly. The CARD-containing prodomain of DRONC is required for interactions with the adaptor molecule DARK (Dorstyn et al., 1999a). The prodomain of DREDD has a death effector domain (DED) and functions in the receptor mediated signalling pathway (Hu and Yang, 2000). The prodomain of STRICA lacks equivalent protein interaction domains but is Ser/Thr rich (Doumanis et al., 2001).

1.3.1 *DCP-1*

Drosophila caspase 1 (*dcp-1*) was the first fly caspase identified based on its homology with the worm *CED-3* gene and has similar substrate specificities to mammalian caspase-3 (Song et al., 1997). Zygotic depletion of *dcp-1* results in a larval lethal phenotype, which includes melanotic tumours and underdevelopment of imaginal discs. Initial studies showed that *dcp-1*/*pita* double mutants, generated by a single P-element insertion, are defective in the transfer of cytoplasmic contents from the nurse cells to the oocyte during oogenesis, termed ‘dumping’, and causing sterility (McCall and Steller, 1998). This phenotype was attributed primarily to cytoskeletal organization failure. A subsequent study employing an EMS mutagenesis screen found mutation of *pita* (the gene flanking *dcp-1*) alone phenocopied the P-element mutant except for the imaginal disc anomaly (Laundrie et al., 2003). This phenotype included a nurse cell ‘dumpless’ phenotype and abnormal nurse cell nuclear morphology. Heat shock activated transgenes in *pita/dcp-1* double mutant animals were used to rescue the phenotype, however, only the *pita*-expressing transgene could rescue the larval lethality, imaginal disc and melanotic tumour phenotypes. *pita*

was also shown to be required for continued follicle cell survival. Because germ cell survival is dependent on follicle cell viability, the double mutants and the *pita* mutants had follicle *and* germ cell defects. The authors then used an imprecise P-element excision strategy to generate a *dcp-1*-specific mutant. This demonstrated DCP-1 was required for stress-induced germ cell death at the mid oogenesis stage, but not follicle cell death at this stage or nurse cell death during late oogenesis (Laundrie et al., 2003). DCP-1 was also shown to be required for normal DRICE activation and localisation in egg chambers (Laundrie et al., 2003).

1.3.2 *DECAY*

DECAY (*Drosophila* Executioner Caspase related to Apopain/Yama) has high sequence homology with the mammalian caspase-3 family of proteins and shares similar substrate specificities (Dorstyn et al., 1999b). *decay* can induce death when overexpressed in cultured *Drosophila* S2 cells and has cleavage activity on the DEVD and VDVAD peptide substrates. While decay transcript is present throughout *Drosophila* development, being high in embryos, in tissues destined to undergo developmental PCD such as the larval midgut and salivary glands, and egg chambers throughout oogenesis, however, its specific role in the cell death pathway and mode of activation remains unclear.

1.3.3 *DAMM*

The smallest identified *Drosophila* caspase has greatest sequence similarity to mammalian caspase-6 (*Mch2*) and is named DAMM (Death Associated Molecule related to Mch2). Although lacking a long prodomain, of the fly caspases, DAMM has greatest homology with STRICA and least with DRONC (Harvey et al., 2001). *damm*

has low expression in the embryo and fails to be upregulated when apoptosis commences at stage 11 of embryogenesis, however, larval salivary glands have abundant transcript as does the adult male and ovaries (Harvey et al., 2001). *damm* overexpression induces death in cultured NIH3T3 cells, to a lesser extent in SL2 cells, and has high VDVAD cleavage specificity. Cotransfection experiments in 293T cells failed to show association of DAMM with DIAP1 or p35 while a dominant negative form suppressed a *hid* overexpression phenotype in the eye (Harvey et al., 2001). The precise physiological function of DAMM *in vivo* remains unclear.

1.3.4 *DRICE*

The effector caspase DRICE, when overexpressed, sensitises *Drosophila* SL2 cells to death stimuli while a truncated form rapidly induces death (Fraser and Evan, 1997; Fraser et al., 1997). DRICE processing is observed in response to RPR- or chemically-induced cell death in *Drosophila* cells (Fraser and Evan, 1997). DRICE cleavage occurs under these conditions and immunodepletion of DRICE in insect cell lysates results in a failure to respond to death stimuli indicating DRICE is essential for apoptosis (Fraser et al., 1997). *drice* is expressed at all stages of *Drosophila* development, is ecdysone-responsive, and is upregulated in the salivary glands prior to their removal indicating a critical role in developmental cell death (Fraser and Evan, 1997; Fraser et al., 1997; Huh et al., 2004b; Yan et al., 2004). Furthermore, abundant DRICE is present in the larval salivary glands and midguts at the time of their destruction (this thesis). Active DRICE is also associated with the process of spermatid individualization (Huh et al., 2004b). Active DRICE is present in mid oogenesis stage (stage 12/13) egg chamber cytoplasm and can be prematurely

induced in stage 8 nurse cell cytoplasm following nutrient deprivation indicating a role for DRICE in nurse cell death (Peterson et al., 2003).

1.3.5 *DREDD*

dredd transcript accumulates in cells fated to undergo PCD and is upregulated in response to ectopic expression of each of the apoptotic activators *rpr*, *hid* and *grim* (Chen et al., 1998). RPR/HID/GRIM-induced apoptosis is suppressed by halving the dose of DREDD indicating its role as a transducer of the death signal when triggered by the *H99* deficiency genes. Interestingly, DREDD processing, while induced by RPR, HID and GRIM, was refractory to certain caspase inhibitors (Chen et al., 1998). DREDD contains a death-inducing domain (DID) which is required for interaction with the *Drosophila* FADD homologue, dFADD, to facilitate DREDD processing (Hu and Yang, 2000).

In response to fungal or bacterial infection, *Drosophila* mount an immune response involving production of antimicrobial peptides and their secretion into the hemolymph (Hoffmann et al., 1999; Lemaitre et al., 1997; Manfruelli et al., 1999). Using an immunocompromised *Drosophila* model in a screen to identify immune response components, DREDD was required for proper immunity in response to bacterial infection (Elrod-Erickson et al., 2000). DREDDs role in a non-apoptotic process was also demonstrated by the observation that antibacterial peptide gene expression was blocked in *dredd* mutants and DREDD was specifically involved in the response to gram-negative bacterial infection (Leulier et al., 2000). DREDD was recently shown to be involved in spermatid individualisation along with dFADD and other core PCD pathway components (Huh et al., 2004b).

1.3.6 *STRICA*

STRICA, like DRONC and DREDD, has a long amino-terminal prodomain, however, while lacking any death effector (DED) or caspase recruitment domains (CARD), it is rich in threonine/serine residues (Doumanis et al., 2001). *strica* transcript is present at low levels at all *Drosophila* developmental stages and overexpression of *strica* in mammalian SL2 cells induces death. This death is marginally repressed by *diap1* coexpression, however STRICA uniquely has been shown to interact with DIAP2 suggestive of a role distinct to that of other initiator caspases (Doumanis et al., 2001). Overexpression of *strica* in the fly eye induces a rough eye phenotype which is reduced by coexpression of *diap1*, *p35* and to a lesser extent, *diap2* (Doumanis, 2004). Genetic and physical interaction between STRICA and DIAP1/2 has been shown, however no equivalent reduction in STRICA-induced cell death in the eye was observed when the *H99* deficiency gene dosage was halved, as occurs with *dronc* overexpression (Doumanis, 2004). A role for STRICA in PCD may be to target DIAP1 for cleavage or degradation. A potential role for the PI3-K pathway component, DAKT, in regulating STRICA-mediated cell killing by phosphorylation of the STRICA prodomain has been suggested.

1.3.7 *DRONC*

DRONC is the only CARD-containing *Drosophila* caspase (Dorstyn et al., 1999a). Initial studies showed DRONC has a substrate cleavage specificity preference for VDVAD (Dorstyn et al., 1999a) and VEID (Hawkins et al., 2000). *dronc* overexpression in NIH 3T3 cells induced cell death, a response abrogated by coexpression of the caspase inhibitor p35. This result was not observed when a

catalytically inactive cysteine mutant (Cys-318-Gly) was expressed (Dorstyn et al., 1999a).

The expression profile of *dronc* is temporally and spatially restricted. Transcript is ubiquitous in early embryos, low through the larval stages, then markedly upregulated at metamorphosis. This upregulation is in response to the late third instar larval and late prepupal ecdysone pulse and occurs in tissues destined to undergo PCD. *dronc* transcript in the midgut rises following the late larval ecdysone pulse initiating histolysis of this tissue over the following 6 h (Lee et al., 2002a). At +12 h RPF, the late prepupal ecdysone pulse induces *dronc* transcription in the salivary glands, after which the salivary glands are rapidly histolysed. Salivary gland removal has been shown to be DRONC-dependent based on studies which overexpress a dominant-negative form of DRONC which has the catalytic cysteine residue mutated (Martin and Baehrecke, 2004). Additionally, *dronc* can be transcriptionally upregulated in response to exogenous ecdysone exposure in the larval midguts and salivary glands (Dorstyn et al., 1999a). *dronc* expression is low in larval eye discs and brain lobes but high in the developing egg chamber preceding nurse cell apoptosis (Dorstyn et al., 1999a).

Overexpression of *dronc* in embryos and in eye discs result in increased TUNEL staining and an ablated eye phenotype respectively, both due to increased apoptosis (Quinn et al., 2000). This eye phenotype, similar to that resulting from *rpr/grim/hid* overexpression, was not observed when the catalytic mutant form of *dronc* was overexpressed in the eye disc, and was reduced by coexpression of *p35* (Quinn et al., 2000). However, it has also been observed that DRONC activity was not suppressed by *p35* coexpression (Hawkins et al., 2000). The phenotype induced by *rpr* or *hid* overexpression in the eye disc is reduced when combined with a *dronc*

deficiency suggesting that DRONC acts downstream of these death activators (Quinn et al., 2000). The adaptor protein DARK is required for the DRONC eye ablation phenotype while the inhibitor of apoptosis proteins, DIAP1 and DIAP2, can reduce this phenotype when co-expressed with DRONC (Quinn et al., 2000). Also, lysates from *dark* hypomorphic mutant flies have reduced DRONC processing indicating an *in vivo* requirement for DARK to achieve maximal DRONC activity. The position of DRONC in the *Drosophila* PCD pathway is shown schematically in figure 1.2.

1.4 Regulation of DRONC activation

diap1 RNAi in S2 insect cells rapidly induces apoptosis, a response prevented by the caspase inhibitor z-VAD and also by pre-treatment with *dronc* RNAi (Muro et al., 2002; Zimmermann et al., 2002). As with *dronc* RNAi pre-treatment, *dark* RNAi also prevents apoptosis induced by DIAP1 ablation (Muro et al., 2002). Western blot analysis shows DRONC processing in response to *diap1* RNAi followed by DRONC-dependent DRICE processing (Muro et al., 2002). The authors postulate that DIAP1 is required to limit the accumulation of a constantly produced DARK-dependent active form of DRONC. A recent study demonstrated the importance of DIAP1 in preventing premature caspase activation in the larval midgut and salivary glands using RNAi transgenes and, while *diap2* is upregulated in salivary glands prior to their removal, its role remains unclear (Yin and Thummel, 2004).

IAPs have been shown to act through their RING finger domain to inhibit RPR-, HID- and DRONC-induced apoptosis (Wilson et al., 2002). In mammals, IAPs can function in diverse cellular roles other than inhibition of caspases, such as signal transduction pathway cofactors and participation in cell division (Bolton et al., 2002; Harlin et al., 2001). IAPs function in part through the presence of up to three

baculoviral IAP repeats (BIR). A C-terminal RING finger domain imparts E3 ubiquitin ligase activity (Li et al., 2002). Mammalian caspase-9 is activated by Apaf-1 and inhibited by XIAP through BIR3 binding (Shiozaki et al., 2003), however, DRONC lacks an equivalent binding motif to accommodate similar binding. The residues flanking the DRONC catalytic cysteine are unique. Most caspases have an active site of a QAC R/Q/K G consensus while DRONC has a PFCRG catalytic consensus sequence (Dorstyn et al., 1999a; Hawkins et al., 2000). Furthermore, DRONC can autoprocess itself and cleave DIAP1 following a glutamate residue, however, it targets the effector caspase DRICE at the orthodox aspartate residue (Hawkins et al., 2000; Yan et al., 2004).

The DRONC binding motif was identified recently to be a 12 residue sequence (residues 114-125) sited between the prodomain and caspase domain, and necessary for BIR2-mediated binding with DIAP1, the BIR1 and RING finger demonstrated no such binding (Chai et al., 2003). This study also showed that a *dronc* mutant (F118E) abrogating DRONC-DIAP1 interactions, maintained its catalytic potential evidenced by a TQTE cleavage assay, and suggests the BIR2-mediated interaction is critical for DRONC inhibition. Furthermore, the interaction of DRONC with the IAP-BIR2 was shown to occupy the same binding pocket as other caspases and the N-terminal region of RPR, HID and GRIM, suggesting a competition between the *H99* deficiency proteins and IAPs exist to regulate DRONC action (Chai et al., 2003). RPR, HID, GRIM and SICKLE were shown to compete out interaction of DRONC with a GST-DIAP1-BIR2 protein (Chai et al., 2003). Earlier work showed that while mutation of the *Drosophila* DIAP1 RING finger failed to prevent interaction with RPR, HID or DRONC, it does completely prevent DRONC ubiquitination resulting from DIAP1 binding (Wilson et al., 2002). Chai *et al* (2003) used an *in vitro*

ubiquitination assay to show that DRONC ubiquitination was dependent on DIAP1-BIR2 and the RING domain, a process prevented by addition of the N-terminal of the pro-death proteins RPR, HID, GRIM or SICKLE. However, the precise role of ubiquitination in the turnover of DRONC *in vivo* remains unclear.

Chai and colleagues (2003) postulate a model whereby DRONC cleavage allows the DIAP1-BIR2 interacting domain to be liberated to functionally sequester and prevent ubiquitination of DRONC by DIAP1 leaving active DRONC to cleave the effector caspases. This mechanism is different to that observed in mammals, whereby IAP interaction with the DRONC analogue caspase-9 renders it catalytically inactive.

1.5 Ecdysone-mediated PCD

The primary transducer of the ecdysone signal in *Drosophila* is the ecdysone receptor heterodimer complex (EcR), composed of the ecdysone receptor and the RXR homologue Ultraspiracle (Usp). When bound by ecdysone, EcR/Usp directly regulates a number of primary response genes (figure 1.4 B) (Burtis et al., 1990; DiBello et al., 1991; Karim and Thummel, 1992). The EcR has three isoforms, each with different expression profiles and biological functions. All three isoforms have Ultraspiracle as its binding partner, an interaction that increases the binding affinity of the hormone to the receptor complex, which in turn stabilises the complex to increase DNA binding affinity (Buszczak and Cooley, 2000; Hall and Thummel, 1998). Steroid hormone receptor superfamily members have two important functional domains, an N-terminal DNA-binding domain containing two zinc-fingers and a C-terminal ligand-binding domain involved in dimerisation and transactivation (Koelle et al., 1991). The

three EcR isoforms, EcR-A, EcR-B1 and EcR-B2, all have identical DNA and ligand binding domains but differ in their N-terminal sequences (Talbot et al., 1993).

Throughout *Drosophila* development, specific EcR isoforms are expressed in tissues with similar developmental fates. Tissues at metamorphosis undergoing proliferation, such as the imaginal discs, have high *EcR-A* and low *EcR-B1* transcript, while most larval tissues which are fated for removal, such as the midguts, express the *EcR-B1* isoform (Talbot et al., 1993). Further support for their respective cellular functions come from isoform distribution analysis in the nervous system. Neurons destined to die predominately express the *EcR-B1* isoform while those remaining functional express the *EcR-A* isoform (Truman et al., 1994). The diversity of function between the EcR isoforms is highlighted when mutant phenotypes are assessed. Animals with a mutation affecting all isoforms are embryonic lethal while *EcR-B1* mutants survive to metamorphosis with defects in tissues that normally have high *EcR-B1* expression, and loss of expression of ecdysone-responsive genes in the larval salivary glands (Bender et al., 1997). Loss of both EcR-B isoforms lead to moulting defects and earlier lethality, but a few animals survive to pupariation permitting the observation that subsets of neurons have remodelling or cell ‘pruning’ defects (Schubiger et al., 1998). Employing heat shock *EcR* rescue transgenes, it was shown that EcR is essential for all larval moults and metamorphosis entry by mediating ecdysone signalling and gene transcription cascades (Li and Bender, 2000). A temperature sensitive *EcR* mutation demonstrated its requirement for normal oogenesis (Carney and Bender, 2000).

1.6 EcR/Usp binding elements and target genes

The requirement or ability of 'early' transcription factors to directly regulate ecdysone-responsive target genes by cooperative promoter binding has been investigated by employing *Drosophila* transgenic for promoter-reporter constructs (Antoniewski et al., 1996; Brodu et al., 1999; Crispi et al., 1998; Crispi et al., 2001; Dubrovsky et al., 2001; Lapie et al., 1993; Laval et al., 1993; Mugat et al., 2000). Using ecdysone-responsive genes as models to investigate how EcR/Usp activates gene transcription, Ecdysone-Response elements (EcREs) were identified in the promoters of these genes, composed of either palindromic or directly repeated half-sites (Antoniewski et al., 1996; D'Avino et al., 1995; Horner et al., 1995). Such binding motifs were shown to be required for quantitative modulation of model transgene expression whereas additional upstream enhancer elements were required to confer the tissue- and stage-specific expression profile (Crispi et al., 2001). Three nucleotide substitutions in the half-site repeats ablated EcR/Usp binding (D'Avino et al., 1995) and promiscuous binding by the βFTZ-F1 orphan nuclear receptor was also prevented (Crispi et al., 1998).

Using the fat body protein as a model gene, an EcRE was shown to be absolutely required for promoter-reporter expression, whereas mutation of another gene-specific EcRE sequence did not alter the stage- and tissue-specific expression, but only the magnitude of its induction (Antoniewski et al., 1996; Crispi et al., 2001). Furthermore, the spacer DNA between EcRE consensus repeats impart variable EcR/Usp binding affinities and it is well documented that *in vitro* binding affinities between EcRE and flanking sequences may deviate from that existing *in vivo*, thus confounding predictions for EcR/Usp binding and its role in regulating different ecdysone-responsive genes.

Employing *BR-C* transcription factor mutants in combination with heat shock induced *BR-C* isoform transgenes, a requirement of BR-C Z3 for reporter activity in the late larval fat body was demonstrated (Mugat et al., 2000). The BR-C Z1 mutant (*rbp*^{5}) had minimal impact on reporter activity.

Brodu et al. (1999) showed a direct and stage-specific requirement of EcR/Usp binding to an EcRE and that an additional and essential factor, dGATAb, binds to specific sites flanking the EcR/Usp consensus. This highlights the requirement of multiple and cooperative tissue- and stage-specific factors for the correct transduction of the ecdysone signal to target genes.

Work using the small heat-shock protein (*hsp*) gene cluster at chromosome position 67B showed these ecdysone-responsive genes to be BR-C dependent and having a BR-C isoform dependent expression profile (Dubrovsky et al., 1994; Dubrovsky et al., 1996). The authors subsequently identified a BR-C dependent DNase 1 hypersensitive site at –1400. This site contains multiple BR-C isoform specific binding consensus sites, one of which requires the direct binding of Z1/Z2/Z3 for correct reporter construct expression, which is abrogated in salivary glands, fat body, imaginal wing discs and midgut when the consensus binding sequence is mutated (Dubrovsky et al., 2001). Relative isoform binding affinities to these sites was demonstrated, further supporting the idea that multiple factors and cooperative activation/repression at promoters modulate the tissue-specific ecdysone response.

Taken together, these data suggest a high complexity to the ecdysone signalling response and indicates that different or multifunctional attributes may be assigned to promoter regulatory elements dependent on the tissue- and stage-specific context and relevant transcription factor abundance.

1.7 The ecdysone-induced genetic regulatory hierarchy

At the onset of metamorphosis the larval midgut and salivary glands show expression of a set of EcR/Usp induced transcription factors that transcriptionally upregulate a host of cell death genes (figure 1.4 B). BR-C, E74, E75, βFTZ-F1 and E93 are transcription factors that play essential roles in the removal of the larval midgut and salivary glands (Broadus et al., 1999; Jiang et al., 2000; Lee and Baehrecke, 2000; Lee et al., 2003; Lee et al., 2002a; Lee et al., 2002b; Lee et al., 2000; Restifo and White, 1991). The relationship of these transcription factors to the core cell death genes such as *rpr*, *hid*, *grim, dark*, *dronc* and *crq*, is complex and differs significantly between the two tissues.

1.8 The ecdysone-induced transcriptional hierarchy in the salivary glands

The steroid triggered transcriptional cascades orchestrating larval salivary gland removal has been rigorously investigated (Ashburner, 1967; Fletcher and Thummel, 1995a; Guay and Guild, 1991; Jiang et al., 1997; Jiang et al., 2000; Kucharova-Mahmood et al., 2002; Lee et al., 2002b; Lee et al., 2000; Martin and Baehrecke, 2004; Yin and Thummel, 2004). The competence factor βFTZ-F1 was required for late prepupal *diap2* expression, *diap2* expression is then repressed by E75 resulting in a temporally restricted burst of *diap2* expression in the late prepupal salivary glands (figure 1.4 B). *rpr* is directly upregulated by EcR/Usp at the late prepupal ecdysone pulse while *hid*, expressed ~1.5 hours later, requires protein synthesis (Jiang et al., 1997; Jiang et al., 2000). *rpr* and *hid* expression is dependent on BR-C Z1 as their expression was dramatically reduced in salivary glands from rbp^{5}

animals while expression of *βFTZ-F1* and *E93* is maintained. Expression of *hid*, but not *rpr*, was dependent on the E74A transcription factor which, when mutated, prevented salivary gland removal in 20% of observed animals (Jiang et al., 1997; Jiang et al., 2000). *EcR*, *BR-C* and *E74A* are all induced in salivary glands at puparium formation however, this does not result in *rpr*, *hid* or *dronc* transcription or histolysis of this tissue, as occurs in the midgut at this time (Lee et al., 2002a).

1.9 Broad-Complex (BR-C)

The *Broad-Complex* (*BR-C*) is located in the ecdysone-inducible early puff at chromosomal position *2B3-5* and has four lethal complementation groups; *broad* (*br*), *reduced bristles on the palpus* (*rbp*), *l(1)2Bc* and *l(1)2Bd* along with a non-complementing set of alleles termed *non-pupariating* (*npr*) (Belyaeva et al., 1980; Kiss et al., 1988; Restifo and White, 1991). *BR-C* encodes a family of DNA binding zinc finger transcription factors formed by temporally and spatially regulated alternative splicing. The four protein isoforms share a common amino terminal core containing a BTB/POZ domain linked to one of four pairs of C2H2 zinc-finger domains, termed Z1 to Z4 (DiBello et al., 1991; Zollman et al., 1994). *BR-C* gene mutation results in variable lethalities, from larval stages in the null mutant, to the early pupal stage in isoform-specific mutants. Animals with the isoform-specific mutations show persistence of the larval salivary glands, visual system organization defects, imaginal disc elongation failures, and CNS maturational abnormalities (Belyaeva et al., 1980; Kiss et al., 1980; Restifo and White, 1991). Each complementation group can be allocated loss of one of the isoforms i.e. *rbp* animals are Z1 isoform mutants, *br* for Z2, and *2Bc* for Z3 mutants (Bayer et al., 1997; Emery et al., 1994). *rbp* (BR-C Z1) mutations are more penetrant regarding salivary gland

persistence compared to *br* (BR-C Z2) and *l(1)2Bc* (BR-C Z3), with 91% and 6% respectively having large intact glands at the early pupal stage (Restifo and White, 1991). The penetrance of proventriculi abnormalities in *rbp*, *l(1)2Bc* and *br* animals was 100%, 93% and 17% respectively (Restifo and White, 1991). Defects in general midgut destruction were mainly restricted to *l(1)2Bc* animals. These data on *BR-C* mutants clearly show variability in the role of different BR-C isoforms between the midgut and salivary glands. While *rbp* mutations primarily affect salivary gland removal, the *l(1)2Bc* mutants affect midgut destruction with the former phenotypes being fully penetrant and the latter having a partial or graded phenotypic range. Studies have also shown BR-C Z1/Z4 and Z2/Z3 isoform function is partially redundant (Bayer et al., 1997).

Analysis of BR-C isoform expression profiles helped to determine whether tissue-specific isoform expression caused tissue-specific developmental changes. Using polyclonal antibodies to the common Br-core region (detecting all protein isoforms), BR-C protein was restricted to the nucleus of multiple larval tissues destined for both proliferation and destruction (Mugat et al., 2000). Staining increased most markedly in salivary glands but also in fat body, midguts, and brain lobes at pupariation following the late larval ecdysone pulse (Emery et al., 1994). Whole animal Western blots showed that Z2 and Z3 protein is maximal at pupariation with Z2 persisting through most of the prepupal period. Z1 reaches maximal levels in the mid prepupal period lasting to around +14 h RPF, the time when salivary gland histolysis occurs (Emery et al., 1994). Z1 protein was the most abundant isoform in the salivary glands peaking at the time of puparium formation and declining through the prepupal stage. Imaginal discs show dynamic BR-C isoform expression switching from Z2 prior to puparium formation to the Z1 isoform late in the prepupal period while

Z3 appears to be the dominant form in the CNS, increasing through the prepupal period (Emery et al., 1994). The fourth isoform, Z4, was identified in an investigation into isoform-specific hormone responses using cultured imaginal discs. In this study, all isoforms were expressed in a primary hormone response, but while Z2, Z3 and Z4 RNA species were high early and dropped over the 6 hours following hormone exposure, the Z1 isoform, due to post-transcriptional processing, peaked only after 6 hours (Bayer et al., 1996). The post-transcriptional modifications determining isoform predominance are thought to be tissue- and stage-dependent as the salivary glands and imaginal discs have different isoform profiles following the late larval ecdysone pulse (Bayer et al., 1996; Emery et al., 1994).

When assessing the ability of the salivary glands to respond to ectopically expressed BR-C isoforms, a study found Z1 delayed pupariation and salivary gland histolysis if overexpressed prior to puparium formation but accelerated salivary gland removal if expressed in the prepupal period (Kucharova-Mahmood et al., 2002). Interestingly, the latter did not alter the timing of head eversion or pupation. Overexpression of Z2 in mid-third instar animals marginally brought forward puparium formation but dramatically induced premature salivary gland histolysis. Z3 overexpression prior to or after puparium formation significantly delayed salivary gland histolysis, however, only overexpression during the prepupal period affected the timing of pupation. Expression of Z4 before puparium formation inhibited pupariation and delayed salivary gland histolysis while expression following puparium formation brought forward pupation and salivary gland destruction.

BR-C mutations affect many gene transcription hierarchies such as loss of induction of the *Sgs-4* intermoult genes and reduced induction of *E74A*, *E75A* and *BR-C* itself suggesting that BR-C is key regulator of the genetic hierarchy at the start

of metamorphosis (Karim et al., 1993; von Kalm et al., 1994). BR-C Z1 isoform is responsible for salivary gland specific transcription of the *L71* group of genes at the prepupal stage, demonstrated by mutation of a *Z1* consensus in a reporter transgene, while the Z3/Z4 isoforms have a repressive action on these genes (Crossgrove et al., 1996).

1.9.1 BR-C-mediated regulation of *dronc* expression

DRONC is necessary for ecdysone-induced apoptosis in the *l(2)mbn* insect cell line (Cakouros et al., 2002). Ecdysone induced expression of the Z1 and Z3 proteins within 2 hours of treatment, followed by the Z4 isoform. Using electromobility shift assays (EMSA) and cold competitor experiments on nuclear extracts from ecdysone treated *l(2)mbn* cells, binding to the BR-C binding consensus sites in the *dronc* proximal promoter was observed by 6 hours in response to ecdysone treatment (Cakouros et al., 2002). Only BR-C Z1 isoform was able to bind and transactivate a *dronc* promoter-luciferase reporter construct in *l(2)mbn* cells. Using RNAi, BR-C was required for ecdysone-induced *dronc* expression and cell death. *In vivo dronc* expression is blocked in *Drosophila* mutant for BR-C Z1 (rbp^5). EMSA experiments using nuclear extracts from rbp^5 animals showed that BR-C Z1 isoform is required to form specific complexes on the *dronc* proximal promoter at relevant developmental stages (Cakouros et al., 2002).

1.10 The role of E93 in PCD

Studies looking for early puff genes that only respond to the late prepupal ecdysone pulse in the salivary glands identified a candidate gene at chromosomal position 93F (Richards, 1976). The 93F puff was different to the other larval puffs due

to its longevity of presence following exogenous ecdysone exposure and it being on salivary gland polytene chromosomes from +12-14 h RPF animals. The 93F puff gene spans a 55kb transcription unit producing a 9.5 kb transcript containing a 3.663 kb ORF which codes for E93, a 146-kDa protein (Baehrecke and Thummel, 1995). *E93* is induced by ecdysone in prepupal salivary glands in the presence of the protein synthesis inhibitor Cyclohexamide indicating it is directly induced. Temporal expression was shown to be the same as the 93F puffing pattern, being highly expressed in the salivary glands only following the late prepupal ecdysone pulse at +10 h RPF. *E93* was transcriptionally upregulated in the midgut of early prepupal animals indicating that *E93* was tightly regulated in a stage- and tissue-specific manner in response to the late larval and late prepupal ecdysone pulses (Baehrecke and Thummel, 1995). The fat body and CNS also show increased expression at pupation. *E93* differed from *BR-C*, *E74* and *E75* in that it did not respond to the late larval ecdysone pulse in the salivary glands, however, it could be prematurely induced by late larval ectopic *βFTZ-F1* expression in this tissue, as could *E74A* and *E75A* (Baehrecke and Thummel, 1995; Woodard et al., 1994).

Elucidation of the role of E93 as a critical regulator of the timing of salivary gland removal and core cell death gene expression came with the isolation of an *E93* mutant (Lee et al., 2000). *E93* mutants show minimal embryological or larval lethality, but fail to contract at pupariation and are early pupal lethal (Lee et al., 2000). *E93* mutant, while developing adult eyes and wings and displaying normal head eversion, have persistent salivary glands, a phenotype rescued by expression of *E93* in the salivary glands demonstrating its requirement for salivary gland histolysis.

E93 protein is present in the midguts and salivary glands prior to their histolysis, is restricted to the nucleus, and not present in tissues which are not

destined for removal (Lee et al., 2000). Polytene chromosomes from +12-14 h RPF salivary glands showed E93 protein localising to early puff and cell death gene loci such as *E74*, *E75*, *E93*, *dredd*, *crq*, *dcp-1* and *drice*. *BR-C*, *βFTZ-F1* and the *H99* deficiency gene loci were not identified as targets (Lee et al., 2000). Northern blot analysis of *E93* mutants confirmed that expression of the *E74*, *E75* and *BR-C* transcription factors was reduced or absent from the salivary glands only at the late prepupal-pupal transition when *E93* is normally expressed (Lee et al., 2002b). The levels of the cell death genes *crq*, *dark*, *rpr*, *hid* and *dronc* were significantly reduced in *E93* mutants while *drice*, *dredd* and *dcp-1* were not detected (Lee et al., 2002b). As with *dronc*, ectopic *E93* expression induces cell death in the wing imaginal disc (Lee et al., 2000). Furthermore, ectopic expression in embryos induces death, but interestingly this death is refractory to p35 expression (Lee and Baehrecke, 2001). These data indicate E93 as a critical regulator of the programmed removal of the salivary glands and the key transducer of the salivary gland hierarchical transcriptional cascade. Figure 1.4 B shows a model of the transcriptional hierarchies acting in the salivary glands at the late larval and late prepupal developmental transitions (Thummel, 1990).

1.10.1 Salivary gland histolysis and its regulation

Recent studies have further exploited the salivary glands as a model for understanding steroid-triggered cell death in *Drosophila* by investigating the role of autophagy and the impact of transcription factor mutations on this process (Lee and Baehrecke, 2001; Lee et al., 2002b).

Following the late prepupal ecdysone pulse ~10 h RPF, salivary glands begin to exhibit typically apoptotic and autophagic changes (Lee and Baehrecke, 2001)

which lead to their rapid destruction approximately 14.5-16 h RPF (figure 1.4 A). As well as DNA fragmentation, nuclear AO staining and membrane phosphatidylserine presentation, dying salivary gland cells also exhibit dynamic vacuolar or 'autophagic' changes. From 12-15 h RPF, salivary gland cells change from cuboidal to a more rounded shape, lose their polytene chromosome banding, and undergo plasma membrane destruction (Lee and Baehrecke, 2001). The large eosin-positive vacuoles change to more numerous and smaller para membranous eosin-negative vacuoles, which can contain cytoplasmic organelles such as mitochondria. By 14.5 h RPF, few vacuoles are observed as the nucleus and cytoplasm condense and finally disperse (Lee and Baehrecke, 2001). As previously described, expression of *p35* in the salivary glands prevents their removal indicating that caspases are required for salivary gland removal (Jiang et al., 1997). However, although the glands do not exhibit DNA fragmentation, they appear to retain some aspects of autophagic vacuolar dynamics, evidenced by the absence of vacuoles in the cytoplasm of persistent TUNEL-negative salivary glands 24 h RPF (Lee and Baehrecke, 2001). Peculiarly, 11% of these persistent glands maintain vacuoles and intact membranes, however, it is likely that vacuolar presence is due to maintenance of membrane integrity.

These results suggest that while caspases are required for normal salivary gland histolysis, aspects of autophagy are independent of caspases. Salivary glands from 24 h RPF *E93* mutants contain large eosin-positive vacuoles, intact nuclei and polytene chromosome banding as do *βFTZ-F1* mutant salivary glands, indicating that typical autophagic changes accompanying salivary gland histolysis have not been initiated (Lee and Baehrecke, 2001). However, while the persistent salivary glands of

E93 mutants have TUNEL staining, βFTZ-F1 mutant salivary glands lack DNA fragmentation (Lee et al., 2002b). Contrasting this in regard to autophagic changes, *BR-C* and *E74A* mutant salivary glands appear to maintain aspects of autophagy evidenced by the absence of vacuoles in mutant salivary glands 24 h RPF indicative of autophagic progression (Lee and Baehrecke, 2001). Furthermore, the persistent glands of *BR-C* and *E74A* mutants have TUNEL-positive nuclei indicating, as well as exhibiting autophagic vacuolar dynamics, that these persistent glands also have the classic hallmark of apoptotic cells, fragmenting DNA (Lee et al., 2002b).

A comprehensive analysis of the transcriptional relationships between the early and late genes in the salivary glands has recently been undertaken (Lee et al., 2002b). This study demonstrates the interdependence of the E93, BR-C and E74A transcription factors with themselves and the core cell death genes, *rpr*, *hid*, *dark*, *crq* and *dronc*, preceding, and over the time of normal salivary gland destruction. *BR-C Z1* mutants have reduced *E93* and no detectable *E74A*, while the *E74A* mutant glands appear to have relatively normal *BR-C* and *E93* transcript levels. *BR-C* mutants have normal *dark*, reduced *rpr*, *dronc* and *crq*, and no *hid* expression. *E74A* mutants have normal *rpr*, *hid* (slightly reduced), *dark* and *dronc*, and reduced *crq*. *E93* mutants have normal *dark*, dramatically reduced *dronc* and no *rpr*, *hid* and *crq* (these transcriptional dependencies are schematically presented in figure 1.4 B) (Lee et al., 2002b).

The transcriptional relationship between βFTZ-F1, E93 and other cell death genes were further explored by employment of a heat shock *βFTZ-F1* transgene in an *E93* mutant background (Lee et al., 2002b). As previously shown, *βFTZ-F1* overexpression induced premature salivary gland destruction and *E93* transcription

(Lee and Baehrecke, 2001; Lee et al., 2002b). E93 was required for this death, but interestingly, variable DNA fragmentation still occurred in these intact salivary glands, indicating that at this stage βFTZ-F1 can bypass E93 and activate aspects of the apoptotic program. The transcription of *rpr*, *crq* and *dronc* are greatly reduced and therefore their transcription is E93-dependent when death is prematurely induced by ectopic *βFTZ-F1* expression in late larval salivary glands. This demonstrates the βFTZ-F1 orphan nuclear receptor is critical in effecting a cell death program in a stage-specific manner.

Recent work investigating links between the molecular mechanisms behind apoptosis and autophagy provided further evidence for caspase function in non-apoptotic aspects of salivary gland destruction and discreet functions regarding substrate targeting (Martin and Baehrecke, 2004). The precise mode by which the salivary glands are removed was unclear, specifically the end stage removal of the dead cells following cytoplasmic engulfment by the lysosomal machinery. In support of previous data, the authors outline the changes in vacuolar dynamics and DNA fragmentation to assess whether non-TUNEL-positive cells contain TUNEL-positive nuclei, an observation which would suggest macrophage engulfment. Vacuoles changed from large to small over +12 -14 h RPF with accompanying detachment from the cytoplasm. TUNEL-positive nuclei were also shown to detach from the surrounding cytoplasm over this time resulting in scattered fragments of encapsulated and condensed cytoplasmic contents by +16 h RPF. No phagocytic engulfment of these scattered salivary gland remnants could be positively identified.

1.11 Caspase substrates and salivary gland destruction

The dynamic cellular changes observed during salivary gland destruction are hypothesised to be, in part, due to specific cytostructural modifications by caspases (Geisbrecht and Montell, 2004; Martin and Baehrecke, 2004). Structural proteins, particularly Lamins, have previously been shown to be caspase targets (Cryns and Yuan, 1998; McCall and Steller, 1998). Martin and Baehrecke (2004) showed that leading up to the destruction of the salivary glands at 15 h RPF, the distribution of α-Spectrin and filamentous Actin changed from diffuse to condensed and was undetectable by +14 h RPF. Lamin was shown to go from nuclear to a cytoplasmic cleaved form, with uncleaved forms undetectable by +14 h RPF. Concomitant with the Lamin dynamics was the appearance of active caspase-3/DRICE in the cytoplasm of +14 h RPF salivary gland cells. The persistent salivary glands of E93 mutants have reduced Lamin cleavage and low levels of active caspases and CRQ, however, α-Tubulin and α-Spectrin do not undergo the changes accompanying WT salivary gland destruction. *BR-C* mutant glands have abnormal α-Tubulin and α-Spectrin distributions but no Lamin cleavage and low active caspase staining. *E74A* mutants have Lamin and caspase activity dynamics similar to WT glands at +14 h RPF but do not have altered α-Tubulin and α-Spectrin.

These results indicate that different levels of caspases in persistent mutant salivary glands contribute toward, or result in, these mutant phenotypes. To identify specific caspases involved in apoptotic or autophagic salivary gland changes, a dominant-negative form of DRONC was expressed in the salivary glands under the control of the fkh-GAL4 driver. 10% of these animals had persistent salivary glands that lacked DNA fragmentation (Martin and Baehrecke, 2004). With particular

relevance to the work in this thesis, midguts from animals expressing a heat shock driven dominant-negative DRONC have TUNEL-positive nuclei indicating DNA fragmentation. This suggests that DRONC exerts fundamental functional differences in the midguts and salivary glands. In persistent salivary glands expressing p35 or dominant negative DRONC, changes in nuclear Lamin DmO, α-Spectrin and α-Tubulin were prevented indicating these protein substrate changes are caspase-dependent. However, changes in filamentous Actin were still observed indicating caspases were not responsible for Lamin cleavage dynamics during salivary gland destruction. Furthermore, the presence of CRQ indicates that aspects of cytoplasmic destruction may also occur independent of caspases, as this protein was cited as a marker for progression of vacuolar dynamics in the dying salivary glands (Martin and Baehrecke, 2004).

1.12 The PCD genetic hierarchy and midgut cell death

The fact that cell death gene transcription is differentially induced in the salivary glands and midguts in response to the late larval and late prepupal ecdysone pulse indicates that the steroid-regulated transcriptional hierarchy differs between these tissues. WT midguts exhibit typical autophagic structures during their destruction (Lee et al., 2002a). These structures include mitochondria and crystalline inclusions within autophagic vesicles and cytoplasmic compaction. Midguts from *BR-C*, *E93* and *E74* mutants all possess TUNEL-positive nuclei but also form adult midgut epithelia demonstrating that some apoptotic and developmental changes are not dependent on these transcription factors. Midgut destruction is most severely affected by mutation of the *BR-C Z3* isoform (*2Bc* complementation group). At +12 h RPF, midguts from these animals appear morphologically similar to +2 h RPF WT

midguts, having persistent proventriculi and gastric caecae. The ultrastructural phenotype of *2Bc* mutant midguts is similar to WT late larval midguts that have intact mitochondria with WT cytoplasmic organization. *E93* mutant midguts at +12 h RPF appear similar to +5 h RPF WT midguts and show regression of the gastric caecae and proventriculus, however, while contraction occurred normally the larval midgut lumen persisted indicating aberrant larval midgut compaction (Lee et al., 2002a). *E93* mutant midguts had ruptured or swollen mitochondria with only some being membrane-bound, suggestive of a partial WT autophagic phenotype. The midguts of *E74* mutant animals appear to undergo destruction normally showing gastric caecae and proventriculus regression, midgut soma contraction and compaction, and intact mitochondria engulfed by autophagic vesicles. While *E93* mutants impact *dronc* transcription, *BR-C* mutants have altered transcription of *rpr*, *hid*, and *crq* but have normal *dark* and *dronc* expression profiles.

The above data demonstrates that salivary glands and midguts utilise core cell death genes to differentially effect apoptosis and autophagy in a stage-specific manner. In addition, the transcriptional hierarchy leading to these changes is tissue-specific in regard to the transcription factors required for apoptotic and autophagic changes and the respective dependence or relationship to the core cell death genes. This demonstrates the importance of an *in vivo* system in exploring steroid-triggered cell death pathways and the requirement for specific caspase mutants to further elucidate their roles in apoptosis.

1.13 Aims

As cell death mechanisms become more fully understood and non-apoptotic roles for caspases begin to be discovered, it is evident that *Drosophila* can be successfully exploited to shed light on mechanisms occurring in humans regarding cell death regulation. The role of hormones in cancer requires greater understanding. In particular, the transcriptional control of the genes involved in its manifestation and progression requires elucidation. At the commencement of this study no caspase mutants in *Drosophila* had been generated and their transcriptional regulation was poorly understood. Therefore, the specific aims of the work described in this thesis were as follows;

1) To generate a *dronc*-specific *Drosophila* mutant and analyse the role of DRONC in developmental PCD.

2) To analyse the promoter of *dronc* to assess its role in regulating the temporally and spatially restricted expression profile of *dronc*.

3) To identify a novel EcR/Usp binding element in the *dronc* promoter and analyse its function in *dronc* expression.

Chapter 2

Materials and Methods

2.1 *Drosophila* stocks and crosses

2.1.1 Genetic Interaction crosses

Experiments testing the genetic interaction of the *dronc* mutant with *GMR-rpr* (White et al., 1996), *GMR-hid* (Grether et al., 1995) and *GMR-grim* (Chen et al., 1996) were carried out at 25°C and eye phenotypes were examined by light microscopy using an Olympus DP11 digital camera.

2.1.2 *dronc* promoter-reporter crosses

For BR-C Z1 isoform mutant (rbp^5) experiments, males homozygous for second or third chromosome *dronc* promoter-*LacZ* transgenes were crossed to virgin rbp^5 heterozygote females balanced with the sex chromosome balancer *Binsn*. The *yellow* mouth hook male larvae, prepupae and early prepupae (hemizygous for the mutated *BR-C* gene and heterozygous for the reporter) were selected for analysis. Control flies are male larvae with normal mouth hooks, which have WT *BR-C* genetic backgrounds (*Binsn/Y*). A similar crossing strategy was employed to analyse reporter activity in the *BR-C* null mutant npr^1.

Fly stocks homozygous for second chromosome 2.8 kb *dronc* promoter-*LacZ* transgenes and heterozygous for the TM6B balancer chromosome and either the *E93* mutant or deficiency ($Df[3R]93F^{x2}$), were crossed and non-TM6B progeny (*DrPr2.8kb-LacZ; E93'/* $Df[3R]93F^{x2}$) were compared to control TM6B progeny (*DrPr2.8kb-LacZ*; $E93^1$ or $Df[3R]93F^{x2}$/ TM6B). A similar crossing strategy was undertaken for *DrPr2.8kb-LacZ*; *E74A* mutant analysis, employing the mutant $E74A^{Pneo}$ and *E74A* deficiency (*Df[3L]st-8IK19*).

The *rbp*^{5} (Restifo and White, 1991), *npr*^{1} (Belyaeva et al., 1980), *E93*^{1} mutant (Lee et al., 2000) and deficiency, and *E74A* (Fletcher and Thummel, 1995b) mutant and deficiency mutant lines were kindly provided by Dr Eric Baehrecke.

2.1.3 *dronc* locus deletion complementation lines

The P-element allele, *KG02994*, was obtained from the Bloomington Stock Centre. Imprecise P-element excision was performed according to standard genetic protocols. Complementation lines were made by cloning a 3.578 kb genomic DNA region covering 1.717 kb upstream of the *dronc* ATG to the *dronc* stop codon into the pCaSpeR-4 transformation vector (Thummel et al., 1988). Insertion of a 'T' at position +20 from the *dronc* ATG was generated to make the *CG6685* complementation construct while insertion of an 'A' at position +33 from the *CG6685* ATG was generated to make the *dronc* complementation construct, both by employment of a standard PCR mutagenesis reaction. Stocks with the imprecise excision third chromosome (named *dronc*^{Z}) were TM6B balanced and crossed into the respective second chromosome complementation transgene lines. Stocks were made homozygous on the second chromosome for either complementation line (named *dronc*^{d5} for animals containing frameshifted *dronc* [WT *CG6685*] and *CG6685*^{4} for animals containing frameshifted *CG6685* [WT *dronc*]) and TM6B/*dronc*^{Z} on the third chromosome. *dronc* and *CG6685* mutants were named *dronc*^{d5} and *CG6685*^{4} respectively.

2.2 *Drosophila* protocols

2.2.1 Staging of animals

For mutant and *dronc* promoter-reporter crosses, approximately fifty virgins and males were left for three days in vials containing standard cornmeal media and then transferred to grape agar lay tubes for staging lays. Staged animals were collected, rinsed briefly in PBS, frozen in liquid nitrogen and stored at -70˚C for assaying or RNA extraction, or dissected and stained as below.

Developmental stages from embryos to third instar larval stages were acquired by ageing animals from time of egg deposition on grape agar plates to desired stages at 25˚C. Third instar larval stages were determined by the gut clearance technique following growth of animals on bromophenol blue supplemented food (Maroni and Stamey, 1983). Pre-pupal and pupal stages were attained by collecting newly pupariated animals from clear gutted third instar larvae populations every hour and ageing at 25^{o}C to desired stages as described (Bayer et al., 1997). Analysis of larval organ cell death and phenotype was performed using clear gut animals.

2.2.2 Germline transformation

Transgenic flies were generated as previously described (Richardson et al., 1995). Briefly, *dronc* promoter-*LacZ* reporter germline transformation constructs (~500ng/μl) and transposase helper plasmid Δ2-3 (~150ng/μl) were microinjected into precellularised *W1118* embryos. w^{+} transformants were screened by eye colour, mapped, and homozygous stocks generated by orthodox techniques.

2.2.3 Lethality tests

Lethality tests were carried out at 25°C. Flies in lay cages were pre-cleared of eggs by changing lay plates three times before collecting embryos from a 4 h lay. Deposited embryos were counted and development to clear gut third instar larvae was monitored for 16 days. All stocks in homozygous lethality tests were balanced with TM6B and larvae were scored as heterozygous or homozygous based on the presence or absence of the Tubby (*Tb*) larval phenotype. The survival rate of homozygous animals was calculated following Mendelian principles of the observed number of heterozygous animals at third instar larval stage determining the expected homozygous complement. Developmental delay was analysed by scoring the emergence of clear gut third instar larvae over the indicated times. Derivation of percentage survival to late third instar stage is as follows; the number of heterozygous third instar larvae counted was taken as being 2/3 of total expected population. The number of observed homozygous animals was expressed as a percentage of expected homozygotes.

2.2.4 Cell death detection

For TUNEL, dissected larval and pupal tissues were fixed for 20 minutes at RT in 4% formaldehyde/0.1% Triton-X in PBST, washed in PBST/ 0.1% Triton-X, washed again in PBST/ 0.5% triton-X and permeabilised by incubation in 100mM Na Citrate/ 0.1% Triton-X at 65°C for 30 minutes. Samples were rinsed in PBST/0.5% Triton-X, incubated for 2 h at 37°C in TUNEL reaction solution in accordance with the manufacturers protocol (In Situ Cell Death Detection Kit, TMR red by Roche), washed in PBST/0.5% Triton-X and mounted in 80% glycerol with 4μg/ml Hoechst for confocal

analysis (BIORAD). For Acridine Orange (AO) staining, larval tissues were dissected in 1.6 μM AO/PBS and incubated for 5 minutes, washed in PBS and analysed by confocal microscopy.

2.2.5 Gamma Irradiation of Larvae

Wandering third instar larvae with full to half-empty guts (see staging by gut clearance technique) were exposed to 8 Gy gamma radiation, allowed to recover at room temperature for 4 h and either dissected for AO staining of tissues or snap frozen in liquid nitrogen and stored at –70°C until required for caspase activity assays.

2.2.6 Caspase cleavage assays

30-50μg of whole animal, larval midgut or salivary gland lysates were incubated at 37°C for 1 h with 50μM caspase substrate in a buffer (50 mM Hepes pH 7.5/ 100mM NaCl/ 1mM EDTA/ 0.1% CHAPS/ 10% sucrose/ 5 mM DTT/ 0.5% Triton-X/ 4% glycerol/ 1X protease inhibitor). Substrate cleavage activity was recorded on a luminescence spectrometer (Perkin Elmer LS 55) using the FL WINLAB software package.

2.2.7 Histology

After coordination of developmental stage, animals were aged to the desired stage of development, the posterior pupal case was punctured with a 26 gauge needle in a drop of FAAG fixative (Campos et al., 1985) (85% etOH/4% formaldehyde/5% acetic acid/1% glutaraldehyde) and nutated overnight at 4°C in the

same fixative. Fixed pupae were rinsed in PBS, paraffin embedded, sectioned and stained using orthodox methods. Images were taken with an Olympus DP11 digital camera.

2.2.8 Detection of β-galactosidase expression

β-Galactosidase (βgal) detection in embryos was carried out essentially as described (Hazelrigg, 2000). Briefly, embryos were collected from grape agar lay plates, dechorionated in 50% bleach/50% PBT, washed in water and fixed for 20 minutes in 50% heptane/50% fixative (0.1 M sodium phosphate, pH 7.5/ 4% formaldehyde) while nutating. Embryos were rehydrated with 0.7% NaCl/0.04% Triton X-100 for 5 minutes, stained with X-gal solution (5 mM K_4 [$Fe(CN)_6$]/ 5 mM K_3 [$Fe(CN)_6$]/ 0.2% X-gal stock [20 mg/ml] in PBS), and incubated at 37˚C for a period dependent on transgene expression levels (usually 3 h). Embryos were devitellinised with 50% heptane/50% methanol by vortexing for 20 seconds, washed with ethanol and mounted in 80% glycerol; 20% PBT for analysis. Adult, pupal and larval tissues were dissected in PBS, fixed for 10 minutes in 2.5% glutaraldehyde/ 50mM Pipes (pH 7.5), washed 3x 5 minutes in PBS, and incubated at 37^{o}C in X-gal staining solution. Tissues were washed in PBS, incubated overnight in 80% glycerol/20% PBS, mounted and photographed.

Images were acquired using Olympus SZ40 microscope set at 3-4x objective with a 2x adaptor lens (110AL2x), fitted with an Olympus DP11 digital camera. Images were cropped and processed using Adobe Photoshop software.

2.2.9 Quantitative β-galactosidase assays

5-10 animals were homogenized in 100μl assay buffer (50 mM potassium phosphate/ 1 mM $MgCl_2$, pH 7.5), the volume made up to 1 ml and vortexed briefly. 10 μl was transferred to 200 μl of chlorophenol red β-D-galactopyranoside (CPRG) solution (1 mM CPRG final concentration) and incubated at 37˚C. βgal activity is represented as the change in A^{574} over 2 h, divided by the sample protein concentration and then multiplied by an arbitrary constant. Control values from *W1118* samples were subtracted to account for endogenous βgal activity.

2.2.10 *Drosophila* cell culture

l(2)mbn cells (kind gift from A. Dorn) (Ress et al., 2000) were grown in Schneider's medium supplemented with 10% FBS. Cells ($2.5x10^6$/ well) were seeded in six well plates in triplicate. Where necessary, ecdysone (10μM) (Sigma) was added for the desired time. Cycloheximide was used at 10μg/ml. Trichostatin A (Sigma) was used at 1μM. Cell viability was assessed by trypan blue exclusion.

2.2.11 Preparation of nuclear extracts and EMSA

$7.5x10^6$ *l(2)mbn* cells were pelleted, washed once in PBS and resuspended in 800μl of buffer A (10mM HEPES pH7.6, 10mM KCl, 1.5mM $MgCl_2$, 0.1mM EDTA, 0.1mM EGTA, Complete™ protease inhibitors from Roche) and placed on ice for 15 minutes NP40 (0.1%) was added and cell suspension vortexed for 30 seconds and centrifuged at 13K for 30 seconds at 4˚C. Nuclear pellets were resuspended in 80μl buffer C (10 mM HEPES pH 7.6, 400 mM NaCl, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, Complete™) and incubated on ice for 40 minutes

while shaking. Extracts were then centrifuged for 5 minutes at 13K and supernatant aliquoted and frozen at –70˚C. Nuclear extracts were prepared from staged larvae ranging through +0-12 h RPF by homogenising 50 larvae in 300μl of buffer A and removed to a fresh tube in a total of 600μl devoid of larval debris. Following incubation on ice for 15 minutes, 10μl of 10% NP40 was added, lysates were vortexed for 30 seconds and spun at 13K for 30 seconds. Nuclear pellets were resuspended in 60-100μl of buffer C and incubated on ice with shaking for 1 h. Lysates were centrifuged for 5 minutes at 13K at 4˚C and supernatant frozen as above. EMSA was carried out by incubating 7-12μg (40μl) of nuclear extracts in binding buffer (20 mM HEPES pH 7.9, 80 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol) containing 1mg/ml BSA and 1μg of poly dI/dC for 5 minutes on ice. Where needed, 2μl of murine EcR-B1, EcR common, EcR-A antibody or a control antibody was also added to the nuclear extracts and incubated on ice prior to the addition of labelled probe. Where indicated 40 ng of cold competitor oligonucleotide was also included. 0.2 ng of labelled probe was added and incubated on ice for a further 20 minutes. 5μl of 5x loading buffer was added and samples electrophoresed on a 5% polyacrylamide/ 0.5xTBE gel. The gel was then dried down onto 3MM Whatmann paper and exposed to Kodak X-ray film.

2.2.12 Immunohistochemistry

Active DRICE staining was carried out essentially as described by Martin and Baehrecke (2004). Briefly, dissected tissues were fixed in 4% paraformaldehyde/PBT for 20 minutes at RT, rinsed 3X 20 minutes in PBT, and blocked in PBSBT (PBS/1% BSA/0.1% Triton-X) for 2 h at RT. Samples were incubated with primary antibodies

overnight at 4°C then washed for 2 h in PBSBT, incubated with secondary antibodies for 2 h followed by 4X 0.5 h washes in PBSBT. Anti active caspase-3 (Cell Signalling Tech.) and anti-Lamin DmO ADL67.10 (Developmental Studies Hybridoma Bank) antibodies were used at 1:50 and 1:400, respectively. Alexaflour-488 and –568 (Molecular Probes) conjugated secondary antibody fluorophores were used to detect DRICE and Lamin respectively. Samples were analysed by confocal microscopy.

2.2.13 Transmission electron microscopy (TEM)

Tissues were dissected from live staged animals in a mild glutaraldehyde/tannic acid fixation solution, fixed 3 h at RT (2.5% glutaraldehyde/ 0.2% tannic acid), post-fixed in 1% osmium tetroxide for 1 h, rinsed in 70% ethanol and then 3X 1 h methanol wash. Samples were then incubated in propylene oxide 0.5 h, propylene oxide/Spurrs' resin 0.5 h, then 4 h in Spurrs' resin. Samples were transferred to caps and placed at 68°C overnight, sectioned and analysed by TEM.

2.2.14 Haemocyte analysis

To assess the haemocyte status of *dronc* mutant animals, wandering third instar *dronc*^{d5} and control larvae were collected and groups of 5 were punctured with a 26-guage needle in a drop of paraffin oil. A 2 μl tip was used to extract the haemolymph exuded from the punctured animals. 0.5-2 μl was made up to 4 μl in 1XPBS, mounted on a haemocytometer slide. Cell fields were recorded by orthodox digital photographic methods.

2.3 *Drosophila* cell line manipulations

2.3.1 Cryopreservation of *Drosophila* cell lines

Cells were harvested and resuspended in fresh medium containing 10% FBS at 5-10 x 10^6 cells/ml. An equal volume of freezing mix (20%FBS/ 10% DMSO/ 70% Schneider's medium) was added to cells in 2ml cryopreservation vials (Nunc). Cells were frozen by controlled rate freezing and stored in liquid nitrogen.

2.3.2 Thawing cryopreserved *Drosophila* cells

Vials of *l(2)mbn* cells removed from liquid nitrogen tanks were thawed at room temperature and seeded into a 25cm^2 flask. Once cells had adhered to the flask after incubation at 27°C, they were washed twice in complete Schneider's medium and incubated in 5ml medium at 27°C.

2.3.3 *Drosophila* cell culture RNA interference (RNAi)

Using the Ambion MEGAscript™ kit, sense and antisense RNA transcripts were synthesised using linearised pCDNA3 or pGEMTEasy constructs as templates. Linearised plasmid DNA was purified by phenol/chloroform extraction following restriction digestion. 1μg linearised plasmid DNA was used in a transcription reaction containing 10x reaction buffer, 50 or 75mM dNTPs (75mM for T7 and 50mM for SP6 transcription) and 2.5 units enzyme mix in a total volume of 25μl. Synthesis reactions were incubated overnight at 37°C. Synthesised RNA was purified by phenol/chloroform extraction and ethanol precipitation. Equal amounts of sense and antisense transcripts were mixed together and secondary structures removed by heating to 65°C for 20 minutes. RNA was cooled slowly to room temperature to

generate dsRNA. dsRNA integrity was analysed on a formaldehyde gel. Insect cells were seeded into tissue culture plates or dishes in the presence of 37nM double-stranded RNA and agitated. Cells were incubated with dsRNA for 48-72 h prior to analysis by PCR, western blotting or death assays.

2.3.4 Transfection and luciferase assay

2μg of pxpGDR2.8kbLuc, pxpGDR2.8kbEcRmutLuc, pxpGDR1.1kbLuc or pxpGDR0.54kbLuc, was transfected using Cellfectin, alone or with 1-5 μg of EcR-B1 expression construct. Equal amounts of DNA were used with the pIE1-4 expression vector. DNA in a total volume of 100 μl in Schneider media (without FBS) was added to Cellfectin (2:9 ratio) in 100 μl total media devoid of FBS and incubated at room temperature for 15 minutes. 800μl of serum free medium was added and then overlayed onto $2.5x10^6$ cells in six well plates. Cells were incubated with the DNA/Cellfectin mixture for 5 h. The medium was replaced by 3 ml of Schneider media supplemented with 10% FBS and cells allowed to recover for 24 h. Where needed, ecdysone (10μM) was added for 24 h. Cells were harvested 48 h post transfection, resuspended in lysis buffer (100mM phosphate buffer pH7.8, 10μM EDTA, 2mM DTT) and frozen three times in liquid nitrogen. After centrifugation for 5 minutes at 13K, supernatant was analysed for luciferase activity. Protein (60-100 μg) was assayed in 200 μl assay buffer (100 mM phosphate buffer, 8 mM $MgSO_4$, 2 mM DTT, 0.75 mM ATP, 0.175 mM coenzyme A) using an illuminometer (Packard).

2.4 Immunoblotting

Protein lysates were prepared from late third instar larvae by homogenisation in lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 % Triton X-100, 1 X protease inhibitors). 150 μg (whole animal) or 50 μg (dissected larval glands) of total protein was electrophoresed on a 15 % SDS-PAGE gel and transferred onto PVDF membrane (Schleicher and Schuell). Purified anti-DRONC polyclonal antibody (Quinn et al., 2000) and anti-DRICE FL serum (a kind gift from B. Hay) was used as previously described (Quinn et al., 2000). Signals were detected by ECF (Amersham Biosciences).

2.5 RT-PCR

Total RNA was extracted from dissected tissues and whole animals using Trizol reagent (Invitrogen) according to the manufacturers protocol. Up to 5 μg of total RNA was used as template for cDNA synthesis in a 15 μl reaction with 200 ng of oligo $d(T)_{18}$ primer using a First Strand Synthesis Kit (Amersham) according to the manufacturers protocol. Using 2 μl of cDNA template, PCR amplification was performed using appropriate primers in a 50 μl reaction employing 22-25 cycles. *Drosophila Rp49* was used as a control. 10 μl of PCR reaction was electrophoresed on a 1.5 % agarose gel for product analysis.

2.6 Standard DNA Manipulations

2.6.1 Quantification of DNA

The concentration of a DNA sample was determined using a spectrophotometer to measure absorption at 260nm, assuming that an $O.D_{260nm}$

reading of 1.0 is equivalent to 50μg/ml of DNA. A scan was also performed to assess the purity of the sample, something which is critical when injecting embryos for transgenesis.

2.6.2 Separation of DNA fragments by electrophoresis

TAE buffer (40mM Tris-acetate, 1mM EDTA) was used to dissolve 0.8%-2% agarose to resolve DNA fragments. 1 x DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the DNA when loading samples. Gels were electrophoresed at 90 volts. Gels were stained in ethidium bromide (2μg/ml) for 10 minutes and visualised under a UV transilluminator (254nm). Alternatively, gels were scanned using a Typhoon 9410 (BIORAD) using ImageQuant software (Amersham Biosciences).

2.6.3 Restriction endonuclease digestion

DNA was digested in 10-100 μl total volume with appropriate restriction enzymes (Amersham Biosciences or New England Biolabs). Reactions contained variable amounts of DNA (depending on application), 0.25-1 unit enzyme/μg DNA, 1x buffer and sterile H_2O. Bovine serum albumen (BSA) was added to 0.1mg/ml when specified. Digests were incubated at 37°C or 50°C (depending on enzyme) for 1–3 h.

2.6.4 Purification of DNA fragments

a) Phenol/chloroform extraction

To purify a DNA sample, the DNA solution was made up to 200μl in sterile H_2O, an equal volume of 1:1 phenol/chloroform added, and the tube vortexed for 30

seconds. The aqueous and solvent layers were separated by centrifugation at 13000 rpm for 3 minutes. The upper aqueous phase was removed and traces of phenol were extracted with an equal volume of chloroform followed by centrifugation as above. DNA was precipitated from the aqueous phase with 2 volumes of absolute ethanol, 1μl glycogen (Roche), and 0.1 volume 3M sodium acetate (NaAc) pH 4.6 and incubated at -20°C for 30 minutes. DNA was pelleted by centrifugation at 13000 rpm for 10 minutes and the pellet washed in 70% ethanol. The pellet was resuspended in an appropriate volume of sterile H_2O.

b) ULTRA-CLEAN™

Digested DNA fragments were routinely purified for further procedures using the ULTRACLEAN™ kit from Geneworks, according to the manufacturer's instructions. Following agarose gel electrophoresis, DNA fragments were excised from the gel overlaying a fluorimager printout. The gel slice was weighed and dissolved in 3 volumes of ULTRA-MELT™ solution at 55°C. ULTRA-BIND™ beads were added to the DNA mix (5μl plus 1μl/μg DNA) and incubated at room temperature for 5 minutes with frequent gentle inversion. The DNA-bound ULTRA-BIND™ beads were pelleted by centrifugation at 13000 rpm for 10 seconds and washed twice in ULTRA-WASH™ solution. The beads were dried at 55°C and the DNA was eluted from the beads by resuspending in 10- 20 μl sterile H_2O. Following centrifugation for 1 minute at 13000rpm, the supernatant containing the DNA was carefully removed and transferred to a clean microfuge tube.

2.6.5 End-filling DNA

Restriction-digested DNA with 3' overhangs was end-filled using Klenow (Amersham Biosciences). A reaction mixture containing 1 unit of Klenow and 0.2mM dNTP mix in 1x One-Phor-All Buffer Plus (Amersham Biosciences) diluted in sterile H_2O was incubated at 37°C for 30 minutes. DNA was then purified by ULTRA-CLEAN™ or phenol/chloroform extraction as described above.

2.6.5 Dephosphorylation of DNA

5' phosphate groups were removed from DNA fragments by treatment with 1 unit of calf intestinal alkaline phosphatase (CIAP, New England Biolabs) in the appropriate restriction enzyme buffer at 37°C for 15 minutes.

2.6.6 Ligation of DNA fragments

10-20μl ligation reactions containing 1 Weiss unit of T4 DNA ligase (USB), 1x ligation buffer (50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 10mM DTT, 50μg/ml BSA, 1mM ATP), 50 ng of vector, insert DNA (1:5 molar end ratio), were incubated at 4°C overnight. 5 μl was heat shock transformed into competent DH5α bacteria.

2.7 PCR amplification and sequencing of DNA

2.7.1 PCR amplification of DNA

a) Taq polymerase amplification

PCR was performed in a 50μl reaction volume containing 1x AmpliTaq reaction buffer (Perkin Elmer), 200μM of each deoxynucleotide-triphosphate (dNTP) (dATP, dTTP, dCTP, dGTP), 2.5mM $MgCl_2$, DNA template (2μl first strand cDNA or 50ng

plasmid DNA), 200ng of each primer, and 2.5 units AmpliTaq polymerase (Perkin Elmer) in sterile H_2O. Standard amplification conditions were as follows; initial template denaturation of 94°C for 4 minutes, 25-32 cycles of 0.5 minutes 94°C denaturation, primer annealing at 55°C 1 minute, and primer extension at 72°C for times dependent on amplified product size. A final 4 minute extension at 72°C was performed.

b) DyNAzyme™ amplification

DyNAzyme™ DNA polymerase (Finnzymes) was used to PCR amplify DNA sequences for cloning into pGEMTEasy™ by T/A cloning. PCR reactions containing 1 x Buffer (10mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton® X-100), 2mM dNTP mix, 200ng of forward and reverse primers, 1 unit of Dynazyme DNA polymerase and 50ng template DNA in a 50μl final volume. PCR conditions used were as above.

c) High fidelity PCR.

For high fidelity PCR amplifications requiring proofreading polymerases the Pwo (*Pyrococcus woesei*) polymerase (Roche) or the Expand High Fidelity PCR System (Roche) were used according to the manufacturers specifications.

2.7.2 Mutagenesis PCR reactions

Single point mutations or insertions in a target DNA sequence were generated using the Quikchange Site-Directed Mutagenesis Kit (Stratagene). 150ng plasmid DNA template was amplified in a 50μl reaction containing 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), 100ng of each mutagenic primer, 2mM each dNTP, and 2.5U *Pfu* Turbo DNA polymerase in sterile H_2O. Reactions were as above but employed only 14 cycles, a 68°C extension temperature and time of 2 min/kb. The methylated parental DNA template was digested with 1μl *Dpn*I restriction enzyme at 37°C for 1 h. 10μl of this reaction was then transformed.

2.7.3 DNA sequencing

Plasmid DNA was sequenced using the ABIPRISM Dye Terminator Cycle Sequencing Reaction Kit. Sequencing reactions consisting of 200ng primer, 5μl BigDye Terminator Ready Reaction Mix Version 3(A/C/G/T-Dye Terminator, dGTP, dATP, dCTP, dTTP, Tris-HCl pH9.0, $MgCl_2$, thermal stable pyrophosphatase, AmpliTaq polymerase) were added to 200ng template DNA and the volume made up to 20 μl with sterile H_2O. The following conditions were used to sequence plasmid DNA in a DNA thermal cycler (Hybaid): [96°C -10 seconds, 50°C -5 seconds, 60°C -4 minutes] x 25 cycles, followed by a 4°C hold. DNA was precipitated by the addition of 1μl glycogen (Roche) and 20μl sterile water/60μl isopropanol, vortexed and incubated at RT for 15 minutes. Sequencing products were centrifuged at 13000 rpm for 20 minutes at 4°C. The pellet was washed in 75% isopropanol, centrifuged at 13000 rpm for 5 minutes at RT, air-dried and sequenced using a Perkin Elmer automated sequencer.

2.8 Harvesting of plasmid DNA from bacterial cultures

2.8.1 Small scale plasmid extraction

2 ml bacterial cultures were grown overnight at 37°C with shaking and pelleted for 1 minute at 2000 rpm. Cell pellets were resuspended in 200μl ice cold Qiagen buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100μg/ml RNase A) and transferred to a 1.5 ml microfuge tube. Cells were lysed by the addition of 200μl buffer P2 (200mM NaOH, 1% SDS). Chromosomal DNA and proteins were precipitated by mixing the lysate with 200μl buffer P3 (3M KAc pH 5.5). Lysates were centrifuged at 13000 rpm for 10 minutes. 500 μl of supernatant was transferred to clean microfuge tubes and DNA was precipitated by addition of 2 volumes (1000μl) 100% ethanol and 0.1 volume (50μl) sodium acetate pH 4.6 and incubated at -20°C for 20 minutes. DNA was pelleted by centrifugation at 13000 rpm for 20 minutes, washed in 70% ethanol and resuspended in 30μl sterile H_2O.

2.8.2 Large-scale plasmid extraction

The QIAGEN plasmid Midi Kit (QIAGEN) was used for large-scale plasmid purification. A 100ml culture was grown at 37°C overnight. Cells were pelleted at 2000 rpm in a BECKMAN Avanti^{TM} J-25 centrifuge and pellets resuspended in 4ml ice-cold buffer P1 (as above). Bacterial cells were lysed by the addition of 4ml buffer P2 (as above). Chromosomal DNA and protein was precipitated by the addition of 4ml buffer P3 (as above) and mixed by inversion several times. Lysates were centrifuged at 15000 rpm for 30 minutes at 4°C. Supernatants were transferred to QIAGEN columns pre-equilibrated with 4ml QIAGEN equilibration buffer QBT (750mM NaCl, 50mM MOPS pH7, 15% isopropanol, 0.15% Triton X-100). The columns were

washed twice with 10ml wash buffer QC (1M NaCl, 50mM MOPS pH7, 15% isopropanol) and DNA was eluted in 5ml elution buffer QF (1.25M NaCl, 50mM Tris-Cl, pH8.5, 15% isopropanol). DNA was precipitated by the addition of 3.5 ml of isopropanol and pelleted by centrifugation at 15000rpm for 30 minutes at 4°C. The supernatant was removed and DNA pellets were air dried and resuspended in 400μl sterile H_2O. The DNA solution was transferred to a 1.5ml microfuge tube and precipitated by the addition of 40μl 3M NaAc, pH 4.6 and 800μl 100% ethanol and incubated at 4°C for 30 minutes. DNA was pelleted by centrifugation at 13000 rpm for 20 minutes, washed with 70% ethanol and resuspended in 50μl sterile H_2O.

2.9 RNA Analysis

2.9.1 Quantification of total RNA preparations

The concentration of RNA was measured by reading the absorbance at 260nm on a spectrophotometer, assuming that an OD_{260nm} reading of 1.0 represents 40μg/ml of RNA. RNA integrity and purity was assessed by an optical density scan and also electrophoresis on a 1% agarose/TAE gel, stained with ethidium bromide, and photographed under UV.

2.9.2 RNA extraction

For extraction of total RNA from adherent cells, cells were scraped off the tissue culture dishes, pelleted by centrifugation in a microfuge and resuspended in 1 ml TRIzol reagent (Invitrogen). For *Drosophila* embryos, larvae, pupae, adult flies or dissected tissues, samples were homogenised using a plastic Eppendorf homogeniser in 100-500 μl TRIzol. The volume of TRIzol was made up to 1ml

following homogenisation. 200 μl chloroform per 1 ml TRIzol was added, shaken vigorously for 15 seconds and incubated a RT for 2 minutes. The sample was centrifuged at 13 000 rpm for 15 minutes at 4°C. The aqueous phase containing RNA was transferred to an RNase-free microfuge tube. If dissected *Drosophila* tissue RNA was being extracted, the Pellet Paint Kit (Invitrogen) was used according to the manufacturers specifications. For whole animals RNA was precipitated by the addition of 500μl isopropanol and incubated on ice for 15 minutes. RNA was pelleted by centrifugation at 13 000rpm for 20 minutes at 4°C, washed with 70% ethanol, air-dried and resuspended in 30-50μl diethylpyrocarbonate (DEPC; SIGMA) -treated sterile H_2O. RNA from dissected tissues was resuspended in 10-15μl DEPC H_2O and 1μl of 20 U/μl RNasin (Promega) added.

2.9.3 RNA gel electrophoresis

10-20μg total RNA in 7.5μl 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 minutes, chilled on ice and 2μl RNA loading dye (50% glycerol, 1mM EDTA pH8, 0.25% bromophenol blue, 0.25% xylene cyanol) was added. 5μl RNA molecular weight markers (Boehringer Mannheim) were denatured and treated as above. Samples were loaded onto a 1.2% agarose gel (1x MOPS {20mM MOPS pH7, 1mM EDTA pH8, 8mM NaAc}, 2.2M formaldehyde in DEPC-treated water) and electrophoresed 'dry' (buffer just covering the gel) in 1xMOPS buffer. Gels were electrophoresed at 80 volts until bromophenol blue dye front had run through 3/4 of the gel. The gel was washed several times in

sterile water prior to scanning on a Typhoon 9410 (BIORAD) using ImageQuant software (Amersham Biosciences).

2.10.4 Northern blotting

a) RNA transfer

Electrophoresed RNA was transferred overnight onto Biodyne nylon transfer membrane (Pall) by orthodox capillary action blotting techniques (Maniatis, 1989). Briefly, the gel was placed face down onto Whatmann paper soaked in 20x SSPE buffer (175.3g/L NaCl, 27.6g/L NaH_2PO_4, 7.4g/L EDTA pH7.4). Nylon membrane, was placed on top of the gel, followed by 3 sheets of Whatmann and a stack of absorbent paper towel. A glass plate and weight was placed on top and the transfer apparatus and left overnight. The filter with transferred RNA was cross-linked using an Ultra-Lum UVC-515 Ultraviolet Multilinker at 1800 Joules/m^2.

b) Probe radio-labelling

DNA probes were labelled with ^{32}P using the Geneworks DNA-gigaprime labelling kit as according to the manufacturer's instructions. Briefly, 30ng of DNA in 11.5μl DEPC H_2O with 2.5μl 10X Decamer Solution was denatured at 95°C for 5 minutes, snap frozen in liquid nitrogen, and thawed on ice. To this mix 5μl 5X Reaction mix, 5μl ^{32}P-dATP (50μCi), and 1 unit of Klenow was added and incubated at 37°C for 7.5 minutes. The labelled probe was purified through Bio-Gel P-6 columns (BIORAD), denatured by incubation at ~95°C for 10 minutes, cooled on ice 1 minute, added to the pre-hybridised filter (see below) and incubated in a HYBAID oven overnight at 65°C.

c) Hybridisation and signal detection

The filter was pre-hybridised in 50ml hybridisation buffer (50% formamide, 5 x SSPE, 1mM EDTA pH8, 5x Denhardts solution, 0.1% SDS, 100μg/ml denatured salmon sperm DNA) at 42°C for 2-3 h. The filter was probed overnight as described. The membrane was washed 2 X 10 minutes in 2x SSC/0.1% SDS and 1 X 5 minutes in 0.5x SSC/0.1% SDS at 65°C. Filter signal was detected on a Typhoon 9410 (BIORAD) using ImageQuant software (Amersham Biosciences).

2.11 Transformation of chemically competent bacterial Cells

2.11.1 Preparation of competent *E. coli* cells

5ml ψ broth (ψb; 20g/L Bacto-tryptone [Difco], 5g/L Bacto-yeast extract [Difco], 5g/L $MgSO_4$, and pH7.6 with KOH) was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. This culture was subcultured 1:20 into 100ml of ψ broth and grown at 37°C with shaking until $O.D_{600nm}$ reached 0.4-0.5. Cells were chilled on ice for 15 minutes and pelleted at 6000 rpm for 5 minutes at 4°C. Media was aspirated and the pellet was resuspended in 40ml ice-cold Tfb I buffer (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 for 5 minutes on ice before being pelleted by centrifugation as above and resuspended in 4ml Tfb II buffer (10mM MOPS, 75mM $CaCl_2.2H_2O$, 10mM KCl, 15% glycerol adjusted to pH 6.5 with 0.5M KOH and filter sterilised). Cells were incubated on ice for 15 minutes. 50μl aliquots were transferred to microfuge tubes on dry ice and stored at -70°C.

2.11.2 Transformation of chemically competent cells

Competent cells were thawed 1 minute on ice. 5 μl of ligation reaction or background control DNA was added to cells, pipetted, and incubated on ice for 20 minutes. Cells were heat shocked at 42°C for 1.5 minutes and placed on ice for 15 minutes. The transformed cells were plated onto agar plates (Luria broth + 15g/L Bacto-agar [Difco]) containing the appropriate antibiotics for plasmid-encoded resistance (ampicillin 100μg/ml, kanamycin 25μg/ml or chloramphenicol 34μg/ml). For blue/white colour selection, plates were spread with 4μl of 1M IPTG (isopropylthio-β-D-galactoside; Progen) and 40μl of 20mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Progen). Plates were incubated overnight at 37°C.

2.12 Protein Analysis

2.12.1 Determining protein concentrations

BCA reagent was used to determine protein concentrations as described by the manufacturers (PIERCE). A standard curve was created by preparing BSA protein standards in the range 0.05-2.0 mg/ml. Reagents A and B were mixed in a 50:1 ratio and 200μl was added to 10μl of each sample and incubated at 37°C for 30 minutes. The optical density at 562nm of each standard was measured and plotted on a graph to obtain a standard curve. This graph was then used to determine the protein concentration of experimental samples prepared as above.

2.12.2 Protein extraction

Whole cell extracts were prepared by resuspending insect or mammalian cells in PBS, to which an equal volume of 2x PLB (100mM Tris-HCl pH 6.8, 200mM DTT,

4% SDS, 0.2 % bromophenol blue, 20% glycerol) was added. Homogenised samples were boiled for 10 minutes prior to analysis by SDS-PAGE. Whole *Drosophila* or dissected tissues homogenised in CPRG assay buffer and quantitated were mixed with 25% volume of 4X PLB, boiled for 10 minutes, centrifuged for 1 minute before SDS-PAGE analysis.

2.12.3 Recombinant protein generation

Transformed colonies of BL21 Star *E. coli* cells were used to inoculate overnight cultures. Cultures were subcultured 1:50 into 200ml LB and grown for 3 h at 37°C with shaking. Protein expression was induced at 37°C for 3 h by the addition of 0.5mM-1mM isopropyl β-D-thiogalactoside (IPTG). Cultures were pelleted by centrifugation at 6000 rpm for 15 minutes and the pellets resuspended in PBS. Cells were lysed by sonication (5 x 1 minute bursts with 2 minutes incubation on ice between each pulse) and clarified by centrifugation at 13000 rpm for 10 minutes at 4°C. For lysates prepared for use in caspase cleavage assays, bacterial pellets were sonicated in Ice Assay Buffer (0.1mM Hepes pH7, 10%PEG4000, 0.1% CHAPS, 10mM DTT, 1X protease inhibitor).

2.12.4 *in vitro* translation

cDNAs were transcribed and translated using the Promega TNT™ Coupled Reticulocyte Lysate System. Protein was labelled with ^{35}S-methionine in a 50μl translation reaction containing 25μl TNT Rabbit Reticulocyte Lysate, 2μl TNT reaction buffer, 1μl T7 or SP6 polymerase, 1μl methionine-deficient amino acid mixture, 4μl ^{35}S-methionine, 1μl RNasin ribonuclease inhibitor, 1μg DNA template and sterile H_2O.

Reactions were allowed to proceed by incubation at 30°C for 90 minutes and were stored at -20°C for up to a week.

2.12.5 SDS-PAGE and protein transfer

Resolving polyacrylamide gels were prepared with the following reagents: 10-15% polyacrylamide solution (BIORAD), 37.5 mM Tris pH8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.05% TEMED 9GIBCO). The resolving gels were cast with Hoefer plates and spacers, and overlayed with a 5% stacking gel (5% polyacrylamide, 0.125M Tris-HCl pH6.8, 0.1% SDS, 0.1% APS, 0.1% TEMED) into which the comb was inserted. A Hoefer minigel tank (Amersham Biosciences) was used to run the gel in protein electrophoresis buffer (25mM Tris, 250mM glycine, 0.1% SDS). Samples were prepared as above, loaded into the wells, and run through the stacking gel at 100 volts, and the resolving gel at 200 volts. The gel was stopped when the dye front reached the bottom of the gel plate. Samples were transferred to a polyvinylidine difluoride (PVDF) (polyscreen Dupont) membrane using a Hoefer semi-dry transfer apparatus (Amersham Biosciences). The transfer apparatus was assembled as follows: two sheets of Whatmann filter paper (2M) pre-soaked in protein transfer buffer (49mM Tris, 39mM glycine, 0.0375% SDS, 20% methanol) were placed on the cathode (+) of the apparatus. The PVDF filter was pre-soaked in methanol and rinsed in tap water and placed on top of the Whatmann, followed by the gel and another 2 pieces of Whatmann. Gel proteins were transferred to the filter for 90 minutes at 130 mAmps.

2.12.6 Western blotting

Filters were blocked in 5% skim milk (Diploma) in PBS-Tween20 (PBS-T) for 3 h at room temperature or overnight at 4°C nutating. Primary antibodies were diluted in blocking solution at various dilutions. Filters were probed with primary antibodies for 2-3 h at room temperature, or overnight at 4°C. Filters were washed 3x 5 minutes and 2x 10 minutes in PBS-T. Alkaline Phosphatase (AP) (Amersham Biosciences) conjugated secondary antibodies were diluted 1:2000 in blocking solution and incubated for 1 h at room temperature, washed as above, and proteins visualised by the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Equal volumes of ECL reagent were mixed and the filter incubated for 3 minutes then scanned on a Typhoon 9410 (BIORAD) using ImageQuant software (Amersham Biosciences).

2.12.7 Stripping Western blots

Filters were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62mM Tris-HCl pH 6.8) for 5-10 minutes at 55°C, shaking gently. Following stripping, the blot was washed twice in PBS-T for 10 minutes and blocked in 5% skim milk/PBS-T as described above for primary antibody probing.

2.13 Plasmid constructs

2.13.1 Generation of *dronc* promoter-reporter *LacZ* constructs

All *dronc* promoter-*LacZ* reporter constructs described in chapter 4 were generated by double insert ligations into the pCasper-4 P-element mediated transformation vector in the following manner. Variable promoter lengths were PCR

amplified from *Drosophila* melanogaster *W1118* genomic DNA using a common reverse primer containing an Nco1 site at the ATG of *dronc*. 5' blunt and 3' Nco1 *dronc* promoter fragments were ligated with 5' Nco1 and 3' Not1 cut *LacZ* into pCasper-4 with a Klenow end-filled EcoR1 and a Not1 site.

For the additional *dronc* promoter-reporter constructs used in chapter 5, regions of the *dronc* promoter were PCR amplified from *Drosophila* genomic DNA using Expand High Fidelity PCR System (Roche) with the primer sets inserting BglII sites at the 5' and 3' ends. PCR products were cloned into pGem-T easy (Promega) and then subcloned into the BglII site of the P-element transformation *LacZ* reporter vector pCaSpeR-NLSLacZ following BglII digestion. Orientation was determined by restriction enzyme digestion and sequencing.

2.13.2 Additional constructs and vectors

a) The luciferase reporter pxpGDR2.8kbLuc contains a 2.8 kb region of the *dronc* promoter upstream of the transcription start site up to the ATG cloned into the luciferase reporter vector, pxpG (kindly provided by P. Cockerill). Deletions of the promoter were made by PCR amplification from 1.1 kb and 0.54kb relative to transcription start site up to the ATG site and cloned into the pxpG luciferase reporter vector.
b) The EcR-B1 expression construct was kindly provided by M. Bender.
c) pcDNA3-DRONC; the coding region of *dronc* was directionally subcloned from pBluescript (Stratagene) into pcDNA3 utilising HindIII/XbaI restriction sites by Dr. Loretta Dorstyn.

d) pUAST, pCaSpeR-4 and pGMR plasmids (Brand and Perrimon, 1993; Richardson et al., 1995; Thummel et al., 1988) were provided by Dr Helena Richardson (Peter MacCallum Cancer Institute, Melbourne).

e) pCaSpeR-NLSLacZ; kindly provided by Dr Carl Thummel.

2.14 Primer list (all primers 5' > 3')

2.14.1 *dronc* promoter-reporter construct primers

DrPrR1Nco1; cgc gga tcc atg gtc cgg ata tgg ctt cca

DrPrR1BglII; cgg aga tct ccg gat atg gct tcc acg cgt

DrPr0.54; gca gag ttc agc gag ttg cga act

DrPrF1.1BglII; gcc aga tct ctg tgt gct aaa ttc ctc aat ta

DrPr1.1; gct ctg tgt gct aaa ttc ctc a

DrPr2.3; cgg gaa cgg gac aat caa aat tca agc gac act

DrPr2.3BglII; cgg aga tct gaa cgg gac att caa aat tca agc

DrPr1.33BglII; cga aga tct aat tgt gat caa cta aag gaa

DrPr1.64BglII; ccg aga tct atg tac gtt atg tta tcg taa gtg ta

DrPr2.8; tga tcc cgc ctg cgc ttt ata

2.14.2 Complementation construct mutation primers

(forward primers only shown; inserted base capitalised)

CG6685mutF; gcg cgg agt ttt tAc ggt tcc ggc gta

droncmutF; ccg ccg gag ctc gTa gat tgg aat gcc

2.14.3 Ecdysone Receptor mutation forward primer

(Substitutions are upper case, the consensus binding site is underlined)

gtt atg caa ttg aga gaa tcc aaa AAc Taa aga ccc aca act ttt tct tac

Chapter 3

Drosophila Caspase DRONC is Required for Multiple Developmental Cell Death Pathways and Stress-Induced Apoptosis

3.1 Introduction

When the work detailed in this thesis commenced, the role of *Drosophila* caspases in PCD was evidenced primarily by assessment of tissue-specific expression profiles, ectopic overexpression, double stranded RNA embryo injections, and *in vitro* tissue and cell culture studies (Chen et al., 1998; Dorstyn et al., 1999a; Dorstyn et al., 1999b; Doumanis et al., 2001; Fraser and Evan, 1997; Fraser et al., 1997; Harvey et al., 2001; McCall and Steller, 1998; Quinn et al., 2000; Song et al., 1997). Generation and analysis of a *dcp-1* mutant demonstrated the potential problem of disrupting genes flanking target genes (Laundrie et al., 2003; McCall and Steller, 1998). This chapter describes the first gene-specific mutation in the *Drosophila* apical caspase *dronc*.

dronc is the only CARD-containing caspase in *Drosophila* and therefore is predicted to be the primary molecule for caspase activation in the fly. DRONC is expressed in a spatially and temporally restricted manner throughout development (Dorstyn et al., 1999a) and is regulated by ecdysone in the larval midgut and salivary glands prior to their destruction (Cakouros et al., 2002; Cakouros et al., 2004; Daish et al., 2003; Dorstyn et al., 1999a; Lee et al., 2002b; Lee et al., 2000). DRONC activation occurs through a CARD-mediated interaction with the adaptor molecule DARK, and DIAP1 directly binds DRONC and prevents its activation by blocking this DRONC-DARK interaction (Goyal, 2001; Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000). Once activated, DRONC binds and activates the effector caspase DRICE (Hawkins et al., 2000; Muro et al., 2002). Recent studies employing caspase RNAi transgenes and a dominant negative DRONC protein have implicated or directly demonstrated an *in vivo* role for caspases in non-apoptotic physiological processes such as spermatid individualisation and also salivary gland cell death (Huh et al.,

2004b; Martin and Baehrecke, 2004). Evidence has also been presented showing DRONC involvement in other non-apoptotic physiological processes such as cell proliferation, migration, and growth (Geisbrecht and Montell, 2004; Huh et al., 2004a; Ryoo et al., 2004).

The aim of this work was to generate and analyse a *dronc* mutant and characterise the role of this caspase in development by assessing viability, developmental cell deaths, and non-apoptotic physiological contexts in which DRONC may have a role. This chapter initially describes the characterisation of a hypomorphic allele of *dronc* generated by insertion of a P-element within the *dronc* promoter and demonstrates a requirement for *dronc* in salivary gland cell death. *dronc* mutant animals were created by a two-step genetic manipulation strategy. *dronc* deletion mutants were generated by excising a P-element from the *dronc* promoter followed by reinsertion of a *dronc* genomic region containing a point mutation in the *dronc* gene, effectively restoring the flanking gene function. This chapter reports that *dronc* is essential for *Drosophila* development and is involved in multiple cell death pathways. It is also required for the removal of the larval salivary glands but is not required for caspase activation in the larval midgut.

Results

3.2 A hypomorphic *dronc* allele

The *Drosophila KGO2994* P-element line has a ~13kb 'Gypsy' vector inserted in the *dronc* promoter 113 bp upstream of the 5' UTR of the *dronc* gene (figure 3.1 A). As *dronc* expression is tightly regulated in specific tissues, this stock was a potential candidate for observing the developmental consequences of disrupted *dronc* expression. *dronc* expression was analysed in the larval midgut and salivary glands, tissues which are destined to undergo programmed elimination during metamorphosis, to assess whether *dronc* expression was significantly disrupted in these tissues and whether this impacted on their programmed removal.

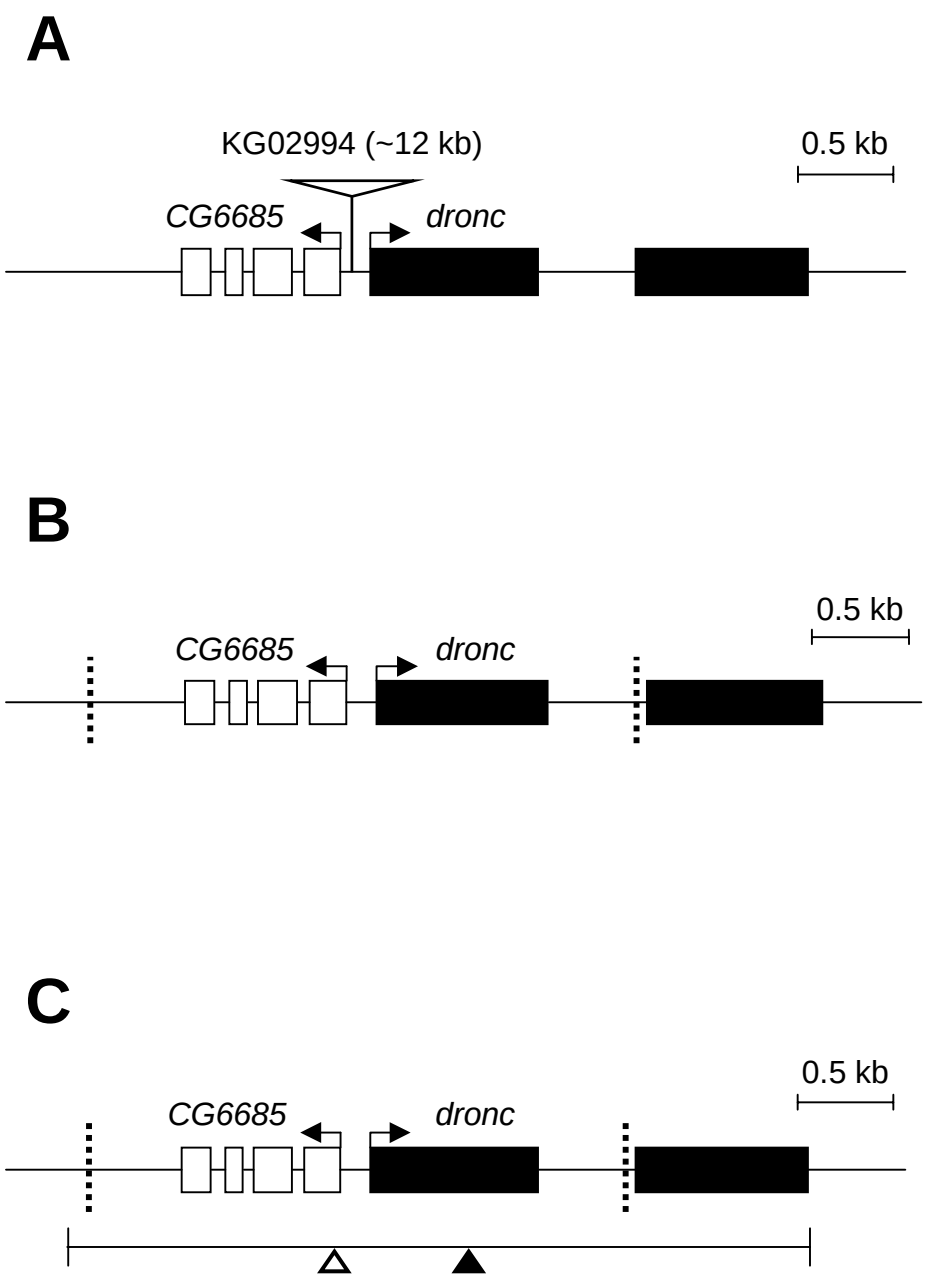
3.3 *dronc* expression in *KGO2994* midguts

As the larval midguts are removed in the prepupal period and is coincident with the time of dramatic *dronc* upregulation in this tissue (Dorstyn et al., 1999a), it was likely that disruption of *dronc* expression at this time would negatively impact midgut destruction. Elimination of this tissue involves shortening of the midgut gastric caeca with a concomitant reduction in midgut length leading to formation of the adult epithelia around the regressing larval structure (Jiang et al., 1997). By 4 h relative to puparium formation (RPF), the proventriculus is markedly reduced in size and the gastric caecae and midgut proper have contracted or regressed (Lee et al., 2002a).

Animals homozygous for the *KGO2994* allele (hereafter referred to as *KGO2994*) and Wild Type (WT) controls were staged relative to the time of puparium

Figure 3.1 the *dronc* genomic region with P-element insertion.

(A) Schematic of the *dronc* locus shows position of the *KG02994* P-element insertion relative to *dronc* and its upstream gene *CG6685*. **(B)** Breakpoints generated by the imprecise excision of *KG02994* (*dronc*^{Z}) are indicated by vertical broken lines. *CG6685* was completely removed along with the first exon of *dronc*. **(C)** Using a 3.578 kb genomic fragment covering this region (horizontal line beneath gene map), complementation constructs were created by introducing frameshift mutations into *dronc* (black arrowhead) or *CG6685* (open arrowhead). Stocks were generated that were homozygous for each respective complementation construct in the imprecise excision background (*dronc*^{d5} has functional *CG6685* and *CG6685*^{4} has functional *dronc*).


A
KG02994 (~12 kb)
0.5 kb
CG6685
dronc
B
0.5 kb
CG6685
dronc
C
0.5 kb
CG6685
dronc

formation and midguts were dissected for RNA extraction and cDNA synthesis. RT-PCR analysis of *dronc* expression in *KGO2994* and control midguts over this period found *dronc* transcript significantly reduced in *KGO2994* animals with a delay of maximal levels compared to controls, which showed abundant *dronc* transcript in the first 2 h RPF (figure 3.2). This demonstrates that the inserted DNA in the *dronc* promoter disrupts the timing and magnitude of *dronc* expression in the midgut.

KGO2994 and WT animals were similarly staged, midguts dissected and homogenized, and the protein run on a gel and transferred for Western analysis. Western blotting for DRONC revealed significantly reduced DRONC protein in *KGO2994* midguts consistent with the RT-PCR data (figure 3.3 A). Very little DRONC protein appears to be present in the midguts of WT and *KGO2994* animals. These animals had guts cleared of blue food, indicating they are within a few hours of puparium formation. In the first few hours following puparium formation, DRONC accumulates in WT but not *KGO2994* midguts and is coincident with the observed increase in transcript levels. No increase in DRONC was observed over the following two hours of prepupal development in the *KGO2994* midguts (figure 3.3 A).

Gross morphological assessment of control and *KGO2994* midguts over the same prepupal period demonstrated a comparable timetable of midgut destruction (figure 3.4 A). WT and control animals were staged as indicated and midguts were dissected and photographed. Gastric caecae were observed to shorten in 2 h RPF WT and *KGO2994* animals while the proventriculus and gastric caecae were no longer present by 6 h RPF in *KGO2994* or control midguts (figure 3.4 A). This

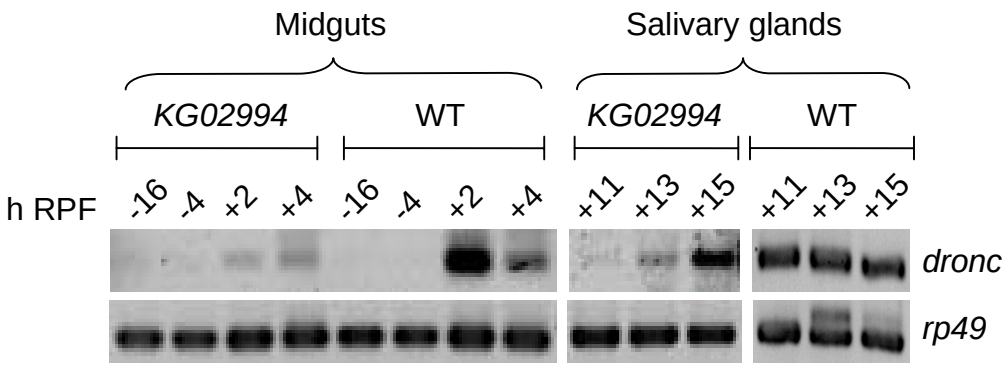


Figure 3.2 RT-PCR of *KG02994* homozygote midgut and salivary gland cDNA.

dronc expression is delayed and reduced in *KG02994* midguts compared to controls that show abundant *dronc* at 2 h RPF. *KG02994* salivary glands show delayed *dronc* transcript peaking at 15 h RPF. Controls show *dronc* is expressed from 11 h RPF.

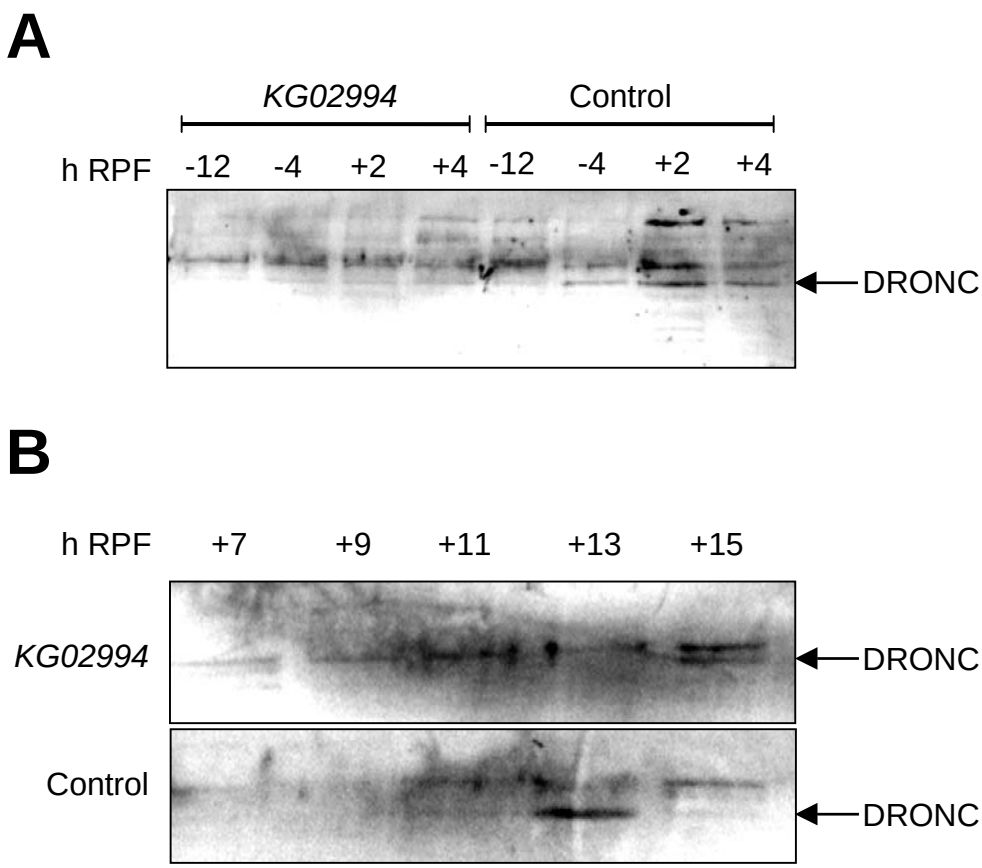


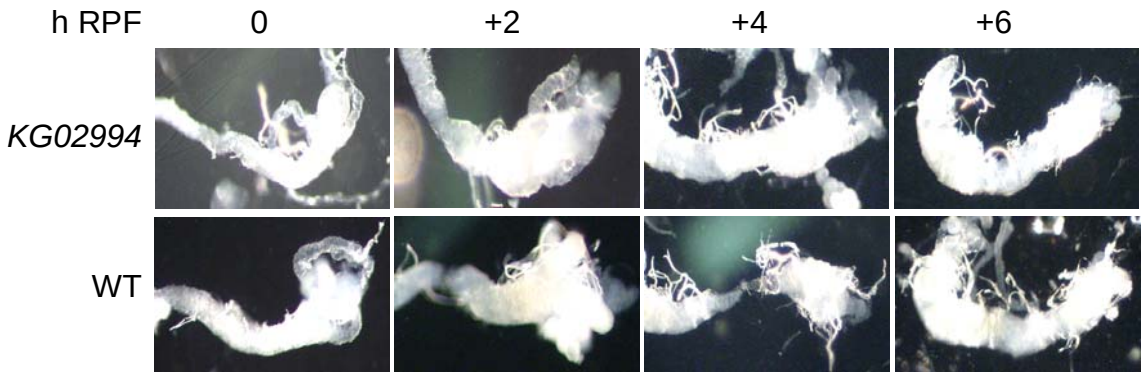
Figure 3.3 *dronc* is deregulated in *KG02994* midguts and salivary glands.

(A) Midguts from staged control and *KG02994* animals were dissected and homogenised for Western analysis. Control animals have abundant DRONC at the start of the prepupal stage (0-2 h RPF) whereas no increase in DRONC is observed in the *KG02994* midguts. **(B)** Control and *KG02994* animals were staged to the indicated times from puparium formation, salivary glands were dissected and protein extracted for Western blotting. DRONC protein increased in +13 h RPF control salivary glands whereas a delayed and lower than WT increase in DRONC is observed in the salivary glands of *KG02994* animals.

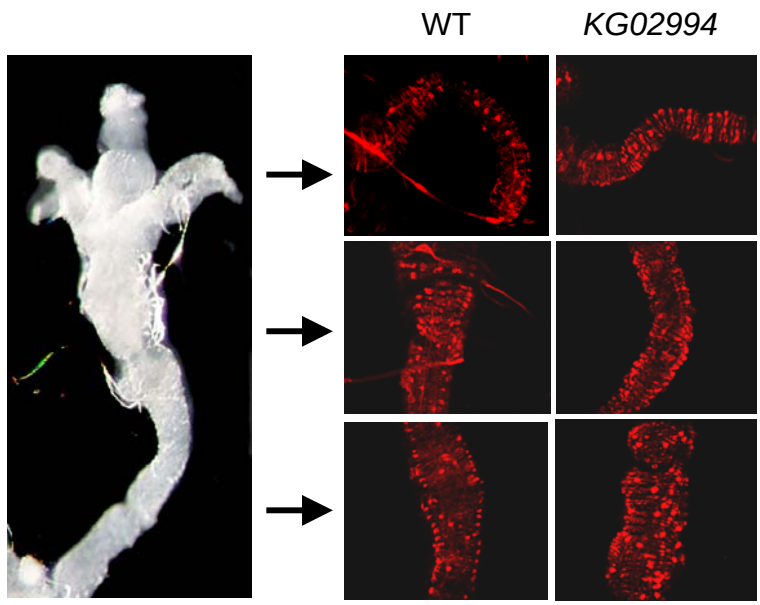
Figure 3.4 Analysis of *KG02994* midgut destruction.

(A) Light microscopic analysis of midgut morphology over the time of larval midgut destruction showing a comparable timetable of midgut histolysis in homozygous *KG02994* animals compared to WT controls. **(B)** TUNEL analysis of *KG02994* prepupal midguts shows no deficit in TUNEL staining evident in any part of the *KG02994* homozygote prepupal midguts compared to controls.

A



B



timetable of midgut destruction is consistent with previous reports (Jiang et al., 1997; Lee et al., 2002a).

Cells dying during midgut removal have fragmenting DNA visualised by TUNEL staining (Jiang et al., 1997). Midguts from WT and *KGO2994* prepupal animals were assessed for DNA fragmentation by TUNEL staining. There were no differences in TUNEL staining in any region of early prepupal midguts or gastric caeca of *KGO2994* animals compared to controls (figure 3.4 B). This result demonstrates that apoptosis can occur in this tissue when DRONC is reduced and delayed.

3.4 *dronc* expression in *KGO2994* salivary glands

dronc is transcriptionally upregulated in salivary glands following the ~10 h RPF late prepupal ecdysone pulse (Lee et al., 2000). *KGO2994* and WT animals were staged to the appropriate developmental times and the salivary glands dissected for RNA extraction and cDNA synthesis. RT-PCR analysis shows *dronc* expression in *KGO2994* salivary glands is delayed with maximal transcript levels occurring ~15 h RPF (figure 3.2). This is later than maximal WT *dronc* transcript levels which occur ~13 h RPF and is consistent with the delay in DRONC in the *KGO2994* salivary glands (figure 3.3 B) (Lee et al., 2002b).

To assess the requirement of DRONC for salivary gland apoptosis, TUNEL staining was performed on *KGO2994* and WT salivary glands prior to the time of their destruction. Salivary glands were dissected from staged WT and *KGO2994* animals, fixed and TUNEL stained for confocal analysis. Salivary glands from *KGO2994*

animals ~14 h RPF showed an absence of TUNEL positive nuclei unlike the WT salivary glands which have TUNEL positive nuclei indicating fragmenting DNA (figure 3.5 A). Nuclear condensation that accompanies PCD was also evident by a reduction in size and number of Hoechst stained nuclei in control glands from 14-15 h RPF animals (figure 3.5 A). Light microscopic analysis of control glands at this time showed a typical contracted morphology progressing in control glands over the ensuing two hours. Haematoxylin and eosin (H & E) stained sections of control and *KGO2994* animals at ~15 and ~17 h RPF demonstrate the histology of dying WT salivary glands and the maintenance of salivary gland structural integrity in *KGO2994* animals at a time when WT glands have been virtually eliminated (figure 3.5 B). The persistence of salivary glands was observed in 33 % (n=87) of *KGO2994* animals at ~17 h RPF compared to control animals (4.5 %, n=44). This hypomorphic phenotype is presumably a consequence of the transcriptional delay in *dronc* expression due to promoter disruption. This suggests that DRONC is required for DNA fragmentation and removal of the larval salivary glands.

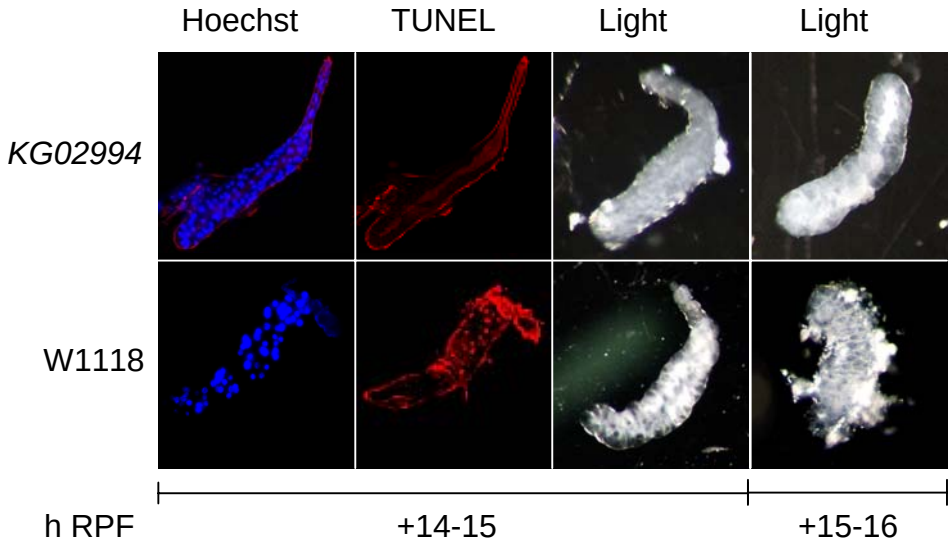
3.5 Creation of a specific *dronc* mutant

To generate an imprecise P-element excision mutant, the *KG02994* line was crossed to a stock containing the *Δ2-3* transposase transgene to mobilize the P-element and excise the flanking *dronc* coding region. Animals in which the P-element was excised and thus were potential *dronc* mutants, judged by the loss of the *white*$^{+}$ gene, were screened by PCR using primers to amplifying the *dronc* genomic region. Three potential *dronc* mutant lines, which amplified an additional smaller PCR product

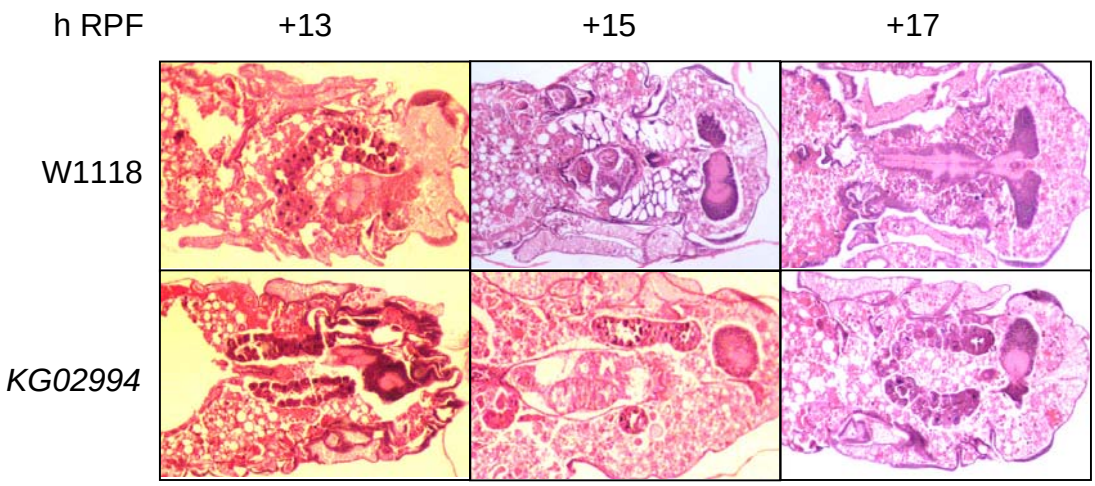
Figure 3.5 *KG02994* animals have aberrant salivary gland destruction.

(A) *KG02994* homozygote salivary glands show a delayed onset of histolysis relative to controls evidenced by maintenance of the Hoechst stained nuclear complement and an absence of TUNEL positive nuclei at +14-15 h RPF. +15-16 h RPF WT glands are contracted and dying while *KG02994* glands remain intact. **(B)** By +17 h RPF, sectioned animals show that control glands have been removed and selected *KG02994* animals have persistent glands. Salivary glands were dissected from animals aged at 25°C to indicated times from point of pupation, dissected and photographed by light microscopy, fixed and TUNEL/Hoechst stained, or fixed, sectioned and stained. (H; Hoechst, T; TUNEL, L; light microscopy, S; H & E stained sections). All developmental stages are shown in hours relative to puparium formation (h RPF).

A


Hoechst
TUNEL
Light
Light
KG02994
W1118
h RPF
+14-15
+15-16

B


h RPF
+13
+15
+17
W1118
KG02994

using primers flanking *dronc*, were identified as containing a deletion (figure 3.7 A shows one of these lines). These lines were further analysed in detail by genomic DNA sequencing. For all three lines the breakpoints were confirmed as being 1926 bp upstream of the *dronc* transcription start site and within the intron of *dronc,* 890 bp downstream from the ATG (figure 3.1 B). One of these deletion lines, named *dronc*^{Z}*,* was chosen for further genetic and phenotypic analysis.

Inspection of the *Drosophila* genome sequence indicated that a gene of unknown function and with no significant sequence homology with other known or predicted proteins, named *CG6685*, was within this deleted region. The developmental expression profile of *CG6685* was assessed. RT-PCR on cDNA from whole animals through development showed that this gene is highly expressed. Abundant *CG6685* transcript is present in early embryos through to the early larval stages, increasing again through the onset of pupation (figure 3.6). No deletion lines isolated were uni-directional excisions, meaning no lines were isolated in which only *dronc* and the not the flanking gene was deleted. Removal of *CG6685* confounded analysis of the *dronc*^{Z} deletion line (see below) regarding DRONC function.

3.6 Generation of complementation transgenes

dronc^{Z} homozygote animals were larval lethal with a host of gross morphological abnormalities (figure 3.8). These animals cease development as empty gut third instar larvae with melanotic tumours (figure 3.9 A). To determine which gene was responsible for the phenotypes observed in *dronc*^{Z} animals, complementation constructs were generated to replace either *dronc* or *CG6685* expression. A region of

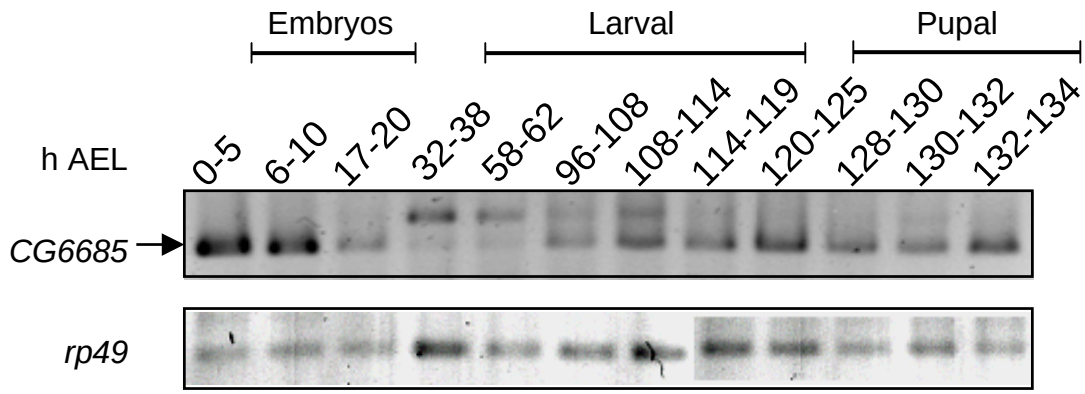


Figure 3.6 Developmental RT-PCR showing expression of *CG6685*.

WT animals were staged to indicated developmental times and RNA extracted for cDNA synthesis. RT-PCR shows the profile of *CG6685* transcript throughout development, showing expression in embryos, low levels in the first and second instars, and appearing to increase at the mid-third instar, late larval and late prepupal ecdysone pulses. Although not quantitative, the expression profile suggests *CG6685* may be regulated by ecdysone.

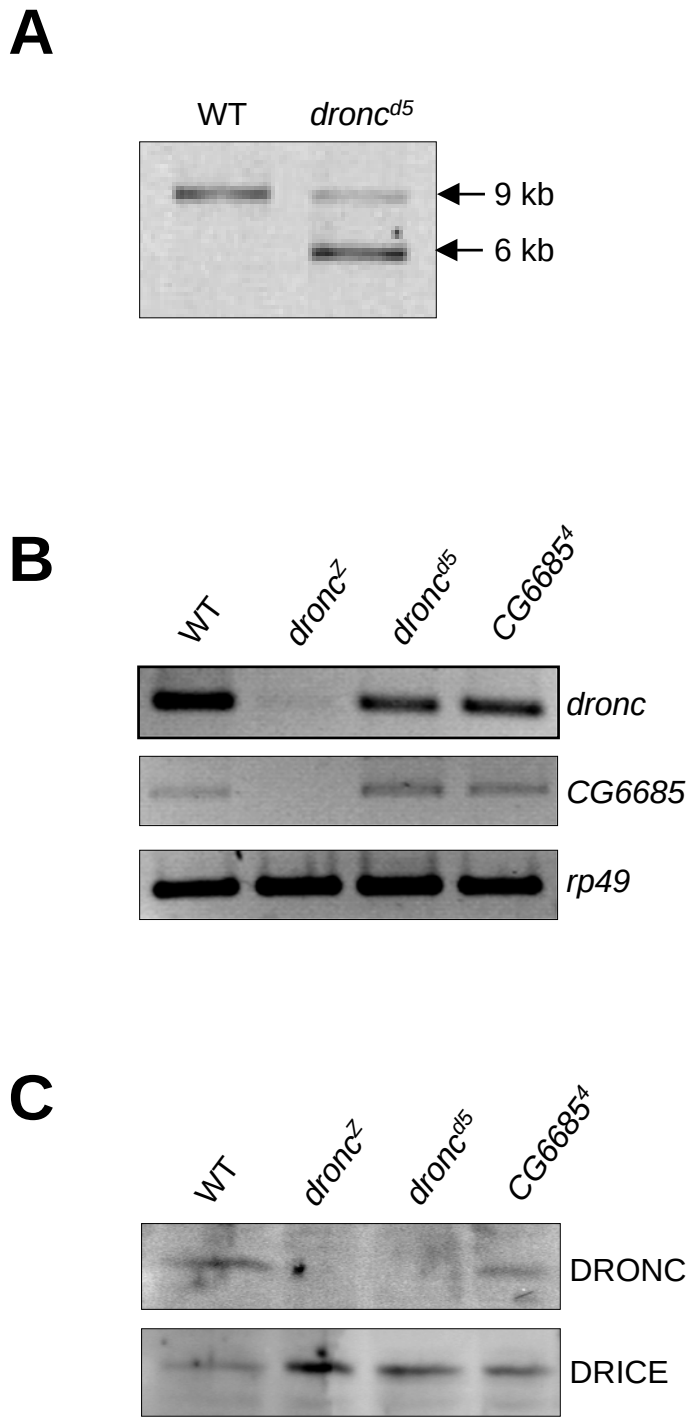
genomic DNA, including promoter sequence previously shown to be sufficient to drive correct stage- and tissue-specific *dronc* expression (Daish et al., 2003), was cloned with frameshift mutations rendering either *dronc* or *CG6685* non-functional (figure 3.1 C) into the pCASPER-4 transformation vector. These constructs were used to generate transgenic flies.

The genetics of the two respective complementation transgenes in the $dronc^{Z}$ mutant background were as followed. Briefly, two 2nd chromosome insertion lines for each complementation construct were selected for analysis in the $dronc^{d5}$ mutant background. Animals homozygous for the *CG6685* frameshift mutation (functional *dronc*) on the 2nd chromosome (figure 3.1 C open triangle) in the homozygous $dronc^{Z}$ deletion background are hereafter referred to as $CG6685^{4}$. Animals homozygous for the *dronc* frameshift mutation (WT *CG6685*) on the 2nd chromosome (figure 3.1 C filled triangle) in the homozygous $dronc^{Z}$ deletion background are referred to as $dronc^{d5}$.

To determine if the transgenes, designed to produce either DRONC or CG6685 protein, were functional and inserted into genomic regions permitting correct transcriptional activity, cDNA was made and protein extracted from third instar animals for RT-PCR and Western analysis respectively. RT-PCR analysis of larval cDNA from WT, $dronc^{Z}$, $CG6685^{4}$ and $dronc^{d5}$ animals demonstrate that transgene transcripts were present (figure 3.7 B). Western blotting with the DRONC antibody confirmed the absence of DRONC in the $dronc^{Z}$ and $dronc^{d5}$ lines (figure 3.7 C). DRONC was present in the $CG6685^{4}$ line, while DRICE levels (used as a control) were unaffected (figure 3.7 C). No antibody for CG6685 was available.

Figure 3.7 Analysis of *dronc* mutant and complementation lines.

(A) Lines with potential deletions generated by the imprecise excision of the p-element were screened by genomic PCR. *dronc*^{Z} samples amplified a second smaller band, 2.816 kb less than the WT chromosome, indicating the deletion size. **(B)** RT-PCR analysis of *dronc* and *CG6685* transcripts in *dronc*^{Z}, *dronc*^{d5} and *CG6685*^{4} whole larvae. Both transgenes have inserted in genomic regions enabling their transcription. **(C)** Immunoblot analysis of DRONC and DRICE in *dronc*^{Z}, *dronc*^{d5} and *CG6685*^{4} whole larvae. DRONC is present in WT and *CG6685*^{4} animals but absent in *dronc*^{Z} and *dronc*^{d5} animals.


A
WT
dronc^d5
9 kb
6 kb
B
WT
dronc^z
dronc^d5
CG6685^4
dronc
CG6685
rp49
C
WT
dronc^z
dronc^d5
CG6685^4
DRONC
DRICE

3.7 *CG6685*^{4} and *dronc*^{d5} animals have different larval organ morphology

WT, *dronc*^{Z}, *CG6685*^{4} and *dronc*^{d5} animals with guts cleared of blue food were dissected in phosphate buffered saline and the central nervous system (CNS), salivary glands, midguts and fat bodies photographed for comparative morphological assessment. *dronc*^{Z}, *CG6685*^{4} and *dronc*^{d5} animals had normal CNS structures. (figure 3.8). However, *dronc*^{Z} larval midguts have shortened gastric caecae and the salivary glands appeared thinner and emaciated. The cells comprising the fat body were detached and rounded as though the cell-cell junctions were failing to maintain a smooth tissue matrix as is present in the WT animals. In *dronc*^{d5} animals that have the functional *CG6685* transgene but lack DRONC, these phenotypes are rescued and the midgut gastric caecae, salivary glands and fat bodies have WT morphology. This phenotypic rescue does not occur in *CG6685*^{4} animals that have the functional *dronc* transgene and frameshifted *CG6685*. This indicates the *CG6685* gene produces a protein involved in organ formation and, as shown below, is required for metamorphosis to occur.

3.8 *CG6685*^{4} and *dronc*^{d5} animals die at different developmental stages

dronc^{Z} animals cease development at the late larval stage with melanotic tumours and developmental defects in some larval tissues (figure 3.8 and 3.9 A). The example shown in figure 3.9 A has a gut cleared of blue pigment normally indicative of animals within a few hours of pupariation. *CG6685*^{4} animals fail to form a normal puparium following gut clearance and arrest as partially contracted larvae with

Figure 3.8 Gross morphology of *dronc* mutant complementation lines.

Larval organs were dissected from animals of indicated genotypes with guts cleared of blue food and photographed. There is a discreet profile of phenotypes associated with the $dronc^{Z}$ and $CG6685^{4}$ animals including shortened gastric caecae, thinner salivary glands, and fat body cell junction anomalies. Replacement of the *CG6685* gene removed these phenotypes from $dronc^{d5}$ animals, which have the functional *CG6685* transgene and non-functional *dronc*.

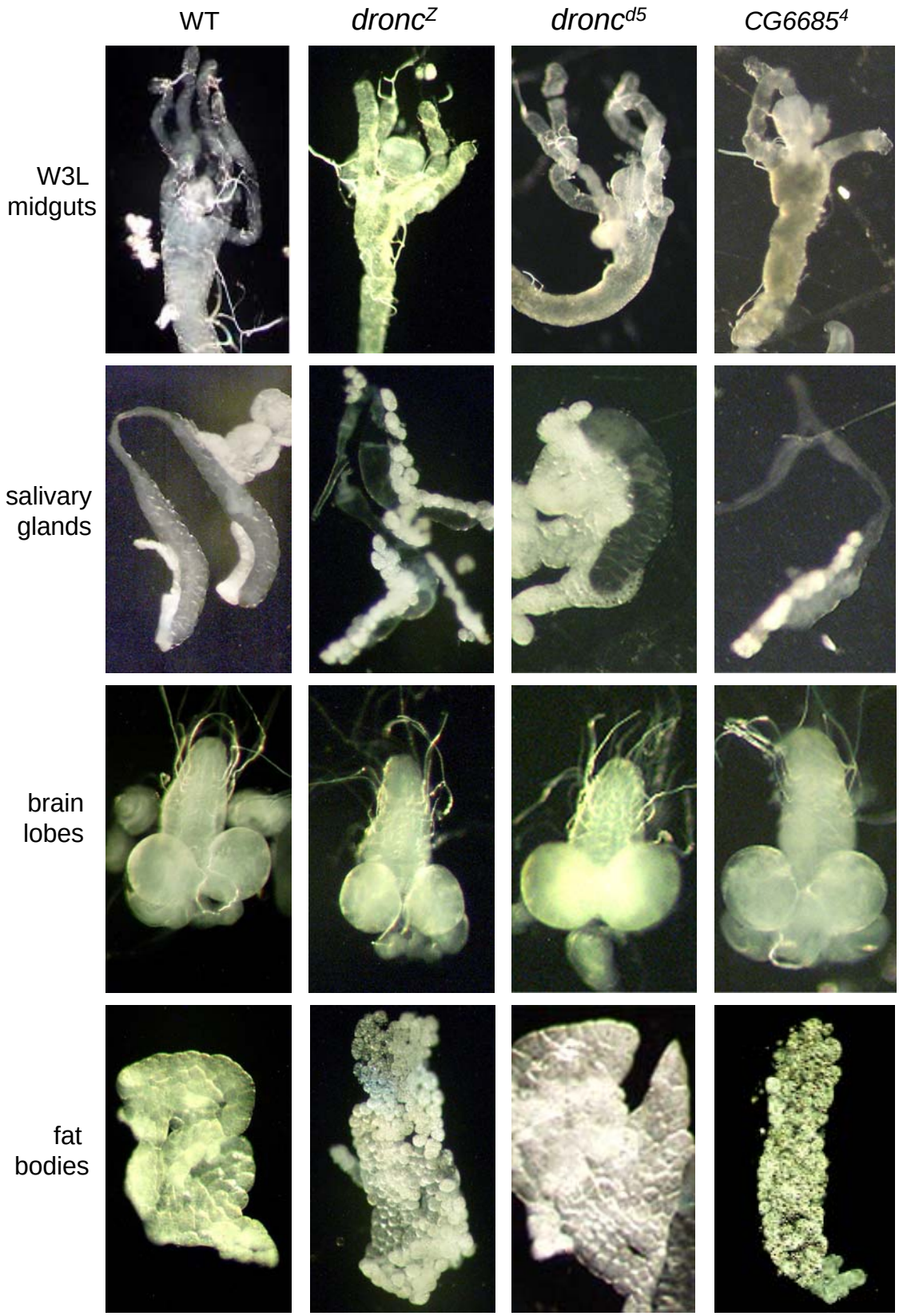

WT
dronc^Z
dronc^d5
CG6685^4
W3L midguts
salivary glands
brain lobes
fat bodies

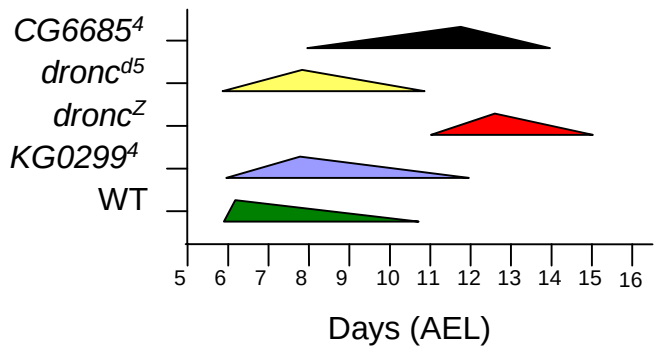
Figure 3.9 Developmental and lethality profiles of *dronc* mutant complementation lines.

(A) Stages of lethality associated with *dronc*^{Z}, *dronc*^{d5}, *CG6685*^{4} and animals containing both complementation transgenes in a *dronc*^{Z} background (*dronc*^{d5}/ *CG6685*^{4}). *dronc*^{Z} and *CG6685*^{4} animals are homozygous late larval lethal, *dronc*^{d5} mid pupal lethal, while 32.67% of expected *dronc*^{d5}/*CG6685*^{4}; *dronc*^{Z} animals survived to eclosion. **(B)** *dronc*^{Z} animals have delayed development that is restored to WT by replacement of CG6685 by the transgene insertion. Peaks indicate the point that the majority of animals reach the third instar larval stage. AEL; after egg laying. **(C)** Assessment of survival rates to third instar of *KG02994*, *dronc*^{Z}, *dronc*^{d5}, and *CG6685*^{4} animals. The error bars represent mean ± SEM for data derived from three independent experiments.

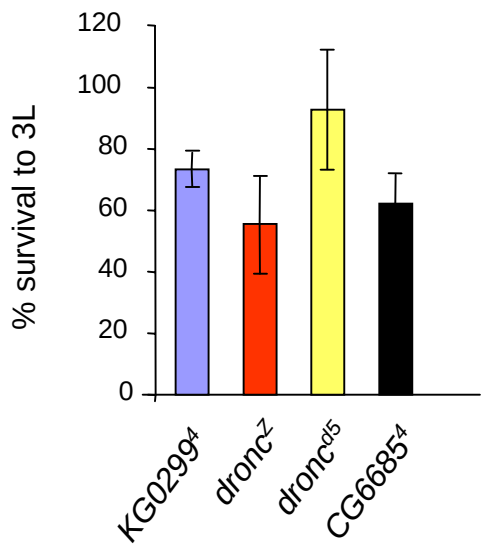
A

dronc^{Z} *CG6685*^{4} *dronc*^{d5} *dronc*^{d5}/ *CG6685*^{4}

B



C



melanotic tumours (figure 3.9 A). *dronc*^{d5} animals pupate normally but arrest as pupae prior to the pharate adult stage (figure 3.9 A). This demonstrates that a critical, but as yet undefined, developmental role is played by the *CG6685* gene product and validates the employment of the complementation transgene strategy.

To further assess the ability of the respective complementation transgenes to emulate the endogenous gene expression profiles and restore viability when combined in the same animal when in a *dronc*^{Z} background, both rescue transgenes were crossed into the *dronc*^{Z} background. *CG6685*^{4}*/ dronc*^{d5}; *dronc*^{Z} animals have dramatically reduced lethality with a survival rate to eclosion of 32.67 % (n=876) of expected (figure 3.9 A) with the remaining non-eclosed animals developing to an advanced pharate adult stage. This result is significant in demonstrating that the discreet phenotypes observed for each complementation transgene when placed in the *dronc*^{Z} genetic background are attributed primarily to the gene product of the non functional gene i.e. DRONC and CG6685 for *dronc*^{d5} and *CG6685*^{4} respectively.

As a detailed expression profile of the *CG6685* transcript throughout *Drosophila* development is not known, it cannot definitively be demonstrated that its correct expression has been restored in *dronc*^{d5} animals. However, the following evidence suggests the phenotype and lethality observed for *dronc*^{d5} animals can be primarily attributed to the absence of DRONC and not disruption to the *CG6685* expression profile; (1) each of the transgenes is associated with discreet developmental defect profiles and survival boundaries, (2) combining the two complementation transgenes in the same animal significantly rescues *dronc*^{Z} lethality along with the individual lethalities of *dronc*^{d5} and *CG6685*^{4}, (3) DRONC is present in

CG6685^{4} animals, (4) *dronc*^{d5} animals have *CG6685* transcriptional activity, and (5) *CG6685* transcript is barely detectable in larval midguts and absent from salivary glands at the time of their destruction.

3.9 *CG6685*^{4} and *dronc*^{d5} animals have different developmental delays and survival rates to the late third instar stage

As there were discreet phenotypes and lethalities associated with the mutation of *dronc* and *CG6685*, the *dronc*^{Z} line was deemed a hybrid phenotype and not informative regarding DRONC-specific function in development. Consequently the mutant phenotypic profiles of *CG6685*^{4} and *dronc*^{d5} were compared to *dronc*^{Z} to further dissect the role of each gene in *Drosophila* development and physiological processes involving regulation of the temporal developmental timetable. This involved analysis of the survival rates of the mutant and complementation lines. There was no significant lethality during embryonic stages, as measured by hatching frequency, in any of the mutant or complementation lines. However, the survival rate of *KG02994* homozygotes to the late third instar stage was significantly decreased to 70% of expected (figure 3.9 C) and eclosion was delayed compared to their sibling heterozygotes. While *dronc*^{Z} and *CG6685*^{4} animals had a reduced survival frequency to the late third instar, this was not observed for *dronc*^{d5} animals (figure 3.9 C). This suggests that the increased larval lethality associated with *dronc*^{Z} and *CG6685*^{4} is due to the loss of *CG6685* gene function and not *dronc*. Additionally, there was observed a reduced developmental delay in *dronc*^{d5} and *KG02994* animals compared

to *dronc*^{Z} and *CG6685*^{4} animals, with the majority reaching late third instar 1.5 days after WT animals (figure 3.9 B).

3.10 Cell death in larval structures is affected by loss of DRONC

In order to investigate any effects the *dronc* mutation (*dronc*^{d5}) was having on larva to cause lethality, organs were analysed for deficiencies in cell deaths by acridine orange (AO) staining. There was a significant reduction in AO staining in the *dronc*^{d5} brain compared to controls, which showed many AO-positive cells (figure 3.10 A-D). AO staining of *dronc*^{d5} larval eye discs revealed a dramatic decrease in cell death compared to controls (figure 3.10 E and F). These data indicates that loss of DRONC results in decreases in cell death in the brain hemispheres and eye discs.

3.11 DRONC is not required for larval midgut destruction

3.11.1 *dronc* mutant midgut destruction occurs at pupariation

As outlined in section 3.2.1, the larval midguts are destroyed by a programmed process of elimination over the prepupal period involving contraction and regression of the anatomical structures. This destruction continued normally in the *KG02994 dronc* hypomorph, which had reduced and delayed DRONC (section 3.2.1). Initial analysis of the role of DRONC in midgut cell death involved confirmation of the absence of DRONC in *dronc*^{d5} midguts. *dronc*^{d5} and WT midguts were dissected from staged animals at the indicated times, homogenised and electrophoresed for Western blotting. There was a complete absence of DRONC protein in *dronc*^{d5} midguts at developmental times when it is abundant in the WT midguts (figure 3.11 A).

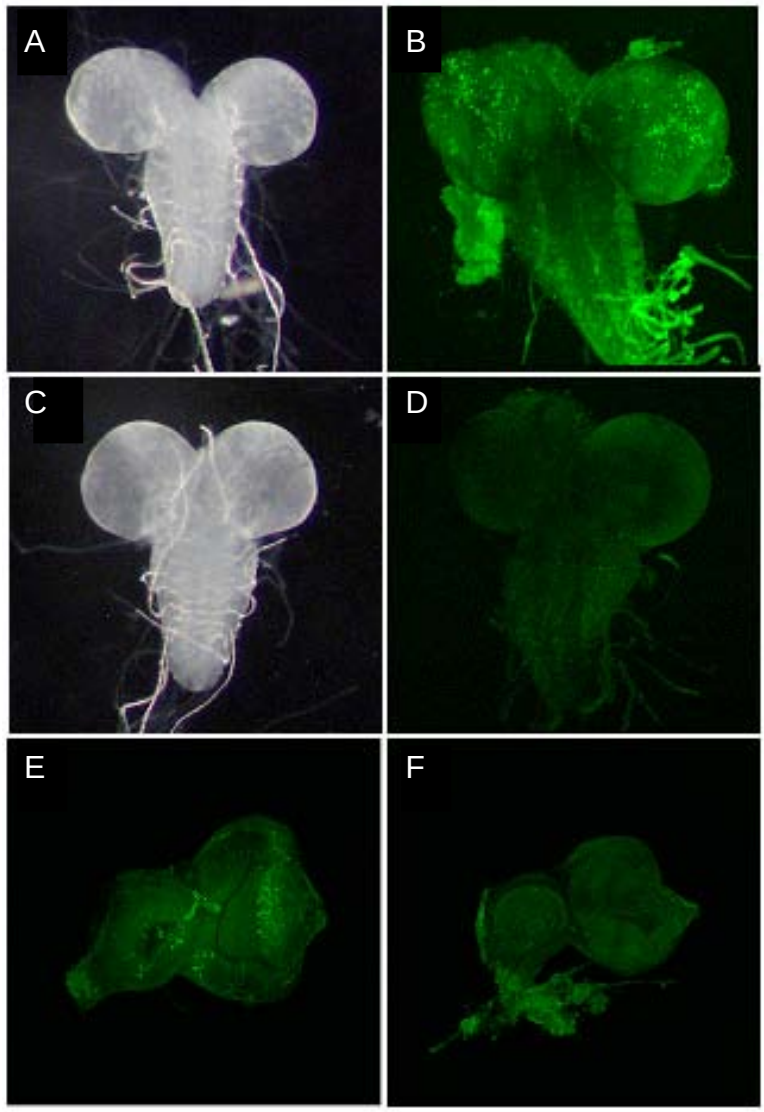
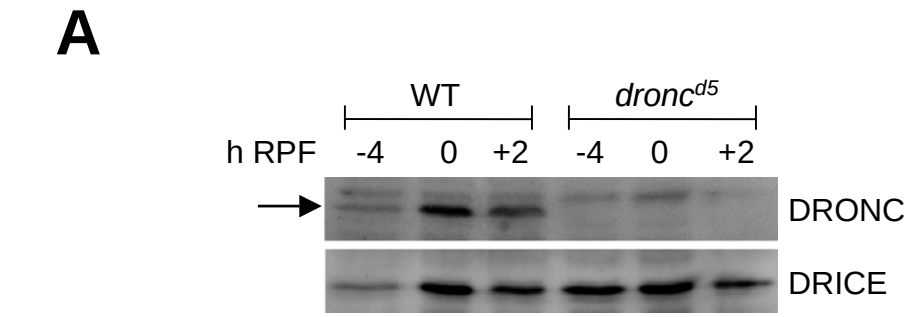


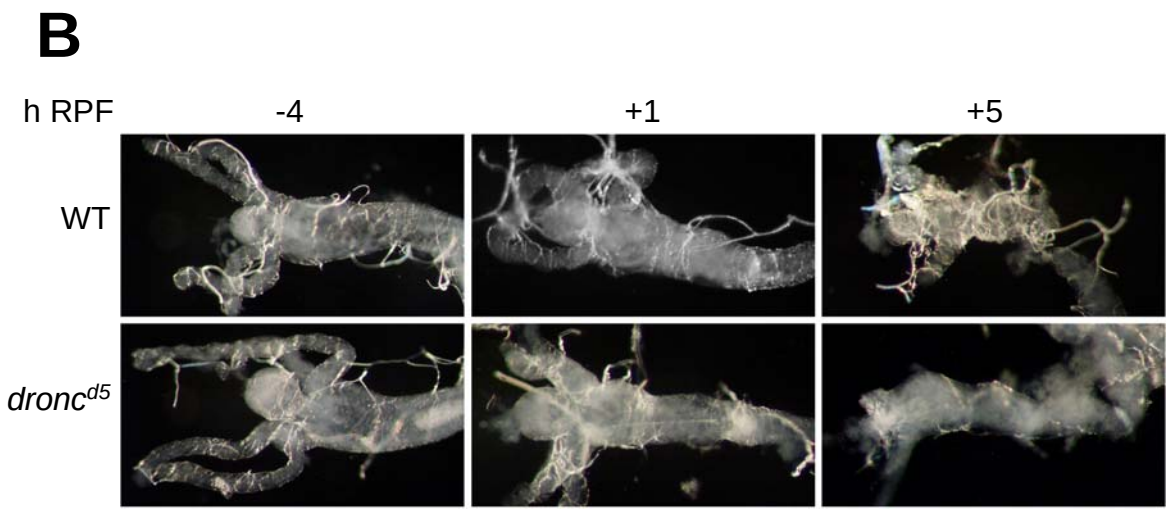
Figure 3.10 *dronc* is required for normal larval organ cell death.

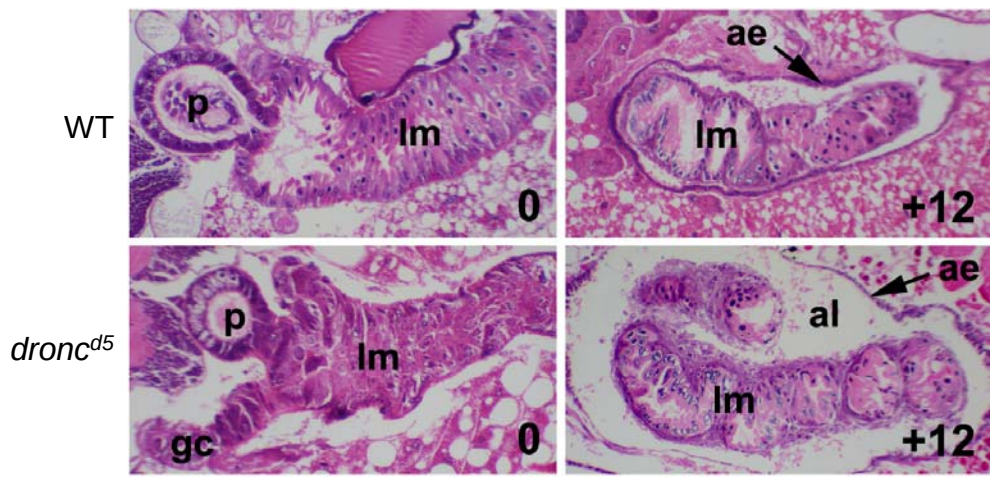
Third instar brain structures and eye discs were dissected from *dronc*^{d5} and control wandering third instar larvae and stained with Acridine orange (AO). **(B and E)** *WT* larval brain lobes and eye discs have many AO positive cells. **(D and F)** In contrast, no significant AO staining is observed in the same structures from *dronc*^{d5} animals. **(A and C)** Brain lobe morphology is similar in *WT* and *dronc*^{d5} larvae.

Figure 3.11 *dronc*^{d5} animals have midguts that undergo destruction.

(A) Immunoblot analysis of *dronc*^{d5} and control midgut protein shows an absence of DRONC in *dronc*^{d5} animals. DRICE is present in dying *dronc* mutant and control midguts. **(B)** *dronc*^{d5} larval midguts undergo destruction similar to WT animals as judged by gross morphology. **(C)** Histological analysis indicates similar extents of larval midgut contraction and compaction in WT and *dronc*^{d5} animals at 12 h RPF (right panels). p, proventriculus; lm, larval midgut; gc, gastric caecae; ae, adult epithelium.


A
WT
dronc^d5
h RPF
-4
0
+2
-4
0
+2
DRONC
DRICE


B
h RPF
-4
+1
+5
WT
dronc^d5


C
WT
dronc^d5
p
lm
0
ae
+12
gc
al

The midguts of similarly staged animals were analysed for defects in midgut destruction by assessment of gross morphology. Within the first six hours of prepupal development, *dronc*^{d5} and WT animals had midguts with shortened gastric caecae, regression of the proventriculus and overall midgut contraction (figure 3.11 B). As previously described, contraction was deemed dependent on formation of the adult epithelia around the histolysing larval midgut, as this phenomenon was maintained in animals with defective core cell death gene transcription as occurs in key transcription factor mutants (Lee et al., 2002a). As can be observed in H & E stained sections from *dronc*^{d5} and WT midguts from 12 h RPF animals, a compact and shortened larval midgut structure is sited within an adult midgut lumen (figure 3.11 C). This adult lumen is encapsulated by the adult epithelia. The larval midgut does not maintain a lumen in *dronc* mutants, as is observed in *E93* mutant animals (Lee et al., 2002a) indicating compaction occurs as in the WT. Furthermore, no anatomical remnants of the larval gastric caecae are present at 12 h RPF in *dronc* mutants (figure 3.11 C). These data are consistent with the *BR-C* mutant midgut phenotype, which has minimal disruption on the level or timing of *dronc* expression. However, as *E93* mutant midguts have significantly reduced *dronc* expression along with defects in compaction, there must be other E93-dependent factors critical for correct execution of this aspect of midgut destruction.

3.11.2 *dronc* mutant midguts are TUNEL positive

Early puff transcription factor mutants, while having variable penetrance regarding larval midgut removal, all maintain midgut TUNEL staining indicating

apoptosis. Consistent with this, late third instar and prepupal *dronc*^{d5} animals have midguts with fragmenting DNA (figure 3.12). Many *dronc*^{d5} gastric caecae and midgut soma cells have TUNEL staining meaning that a DRONC-independent programmed cell death mechanism is active in this tissue either in the absence of, or independent to DRONC.

3.11.3 Caspase activation is maintained in *dronc*^{d5} midguts

To further investigate the prospect of DRONC-independent caspase activation, caspase activation and substrate cleavage levels were analysed in prepupal WT and *dronc*^{d5} midguts. Western blotting demonstrated that *dronc*^{d5} midguts had abundant DRICE protein at late larval and prepupal stages similar to that present in WT midguts (figure 3.11 A).

As apoptosis requires the activation of caspases to cleave target substrates, a caspase cleavage assay was performed on dissected WT and *dronc* mutant midgut homogenates using VDVAD-amc fluorogenic peptides. WT midguts had high activity at the late larval stage with levels dropping marginally over the following four hours of prepupal development (figure 3.13 A). Surprisingly, *dronc* mutant midguts maintained a similar, if not with increased levels, caspase activity profile over the same developmental stages (figure 3.13 A). This indicates that a DRONC-independent mechanism leading to caspase activation occurs in the midgut.

To assess whether DRICE/DCP-1-like effector caspases are being activated in the midguts of *dronc* mutants to cause PCD in the absence of an apical caspase, an anti-active caspase-3 antibody was employed. Immunohistochemical staining with this

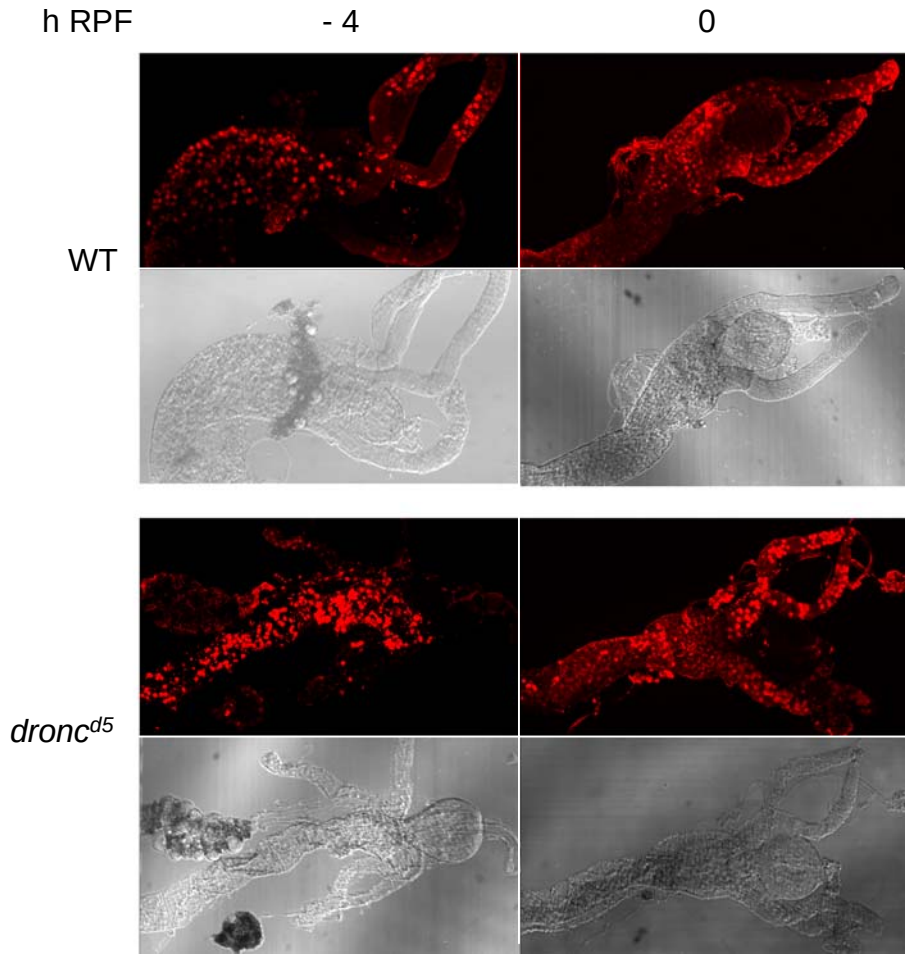


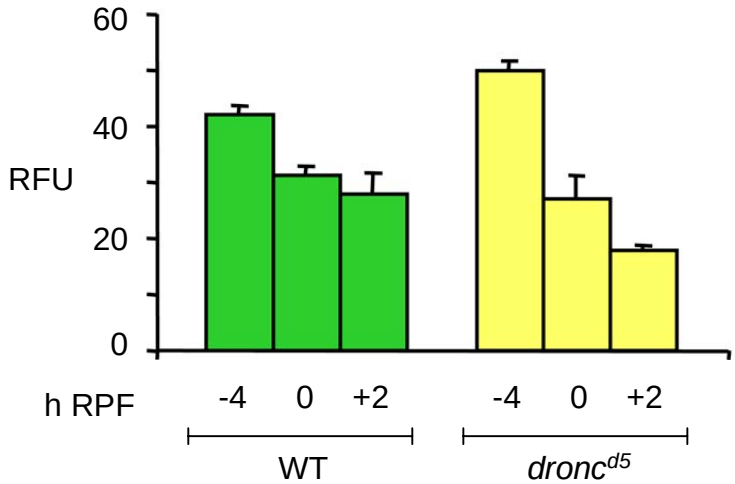
Figure 3.12 TUNEL staining of WT and *dronc*^{d5} midguts.

Normal midgut cell death was observed in *dronc*^{d5} animals. No difference in the late larval (–4 h RPF) or early prepupal (0 h RPF) TUNEL staining was detected between *dronc*^{d5} and control midguts, both showing widespread staining throughout the gastric caeca and midgut.

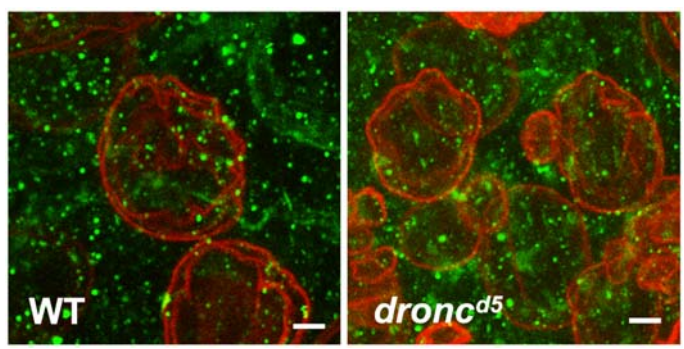
Figure 3.13 Caspase activity is maintained in *dronc*^{d5} midguts over the time of midgut destruction.

(A) Caspase activity was measured on VDVAD-AMC substrate and expressed as relative fluorescence units (RFU). Caspase activity is high in both WT and *dronc*^{d5} midguts. Values represent the mean ± SD derived from 3 independent experiments. **(B)** Confocal images of WT and *dronc*^{d5} midguts immunostained for active DRICE (green) and nuclear Lamin (red). In control experiments, no background staining was seen with the secondary antibodies used here. Scale bars in B equal 2 μm.

A



B



antibody showed active DRICE present in both mutant and control prepupal midgut cells (figure 3.13 B).

The fact that DNA fragmentation and caspase activity precedes the time of maximal *dronc* transcription indicates that apoptosis is initiated in the absence of this apical caspase in the WT animal. Given the abundance of *dronc* transcript and protein in prepupal WT midguts, it is surprising that larval midgut destruction continues in the complete absence of DRONC. The non-requirement of DRONC in the process of midgut histolysis is further supported by the observation that considerably high midgut caspase activity remained in the absence of DRONC.

3.11.4 Aspects of autophagy are absent in *dronc*^{d5} midguts

The ultrastructural autophagic changes occurring in prepupal midgut cells have been documented and include membrane-bound mitochondria and cytoplasmic inclusion bodies (Lee et al., 2002a). As with gross morphology, transcription factor mutants also have variable phenotypes regarding autophagic changes. Transmission electron microscopy was employed to determine if autophagy is affected in *dronc*^{d5} prepupal midguts. Figure 3.14 shows that typical features of autophagy are present in WT midguts, including membrane bound structures such as crystalline inclusion bodies. However, not all autophagic features appear to be present in the *dronc*^{d5} prepupal midgut cells. While early stage small autophagic vacuoles and crystalline inclusion bodies are abundant, large double membrane-enclosed structures were rarely seen (figure 3.14).

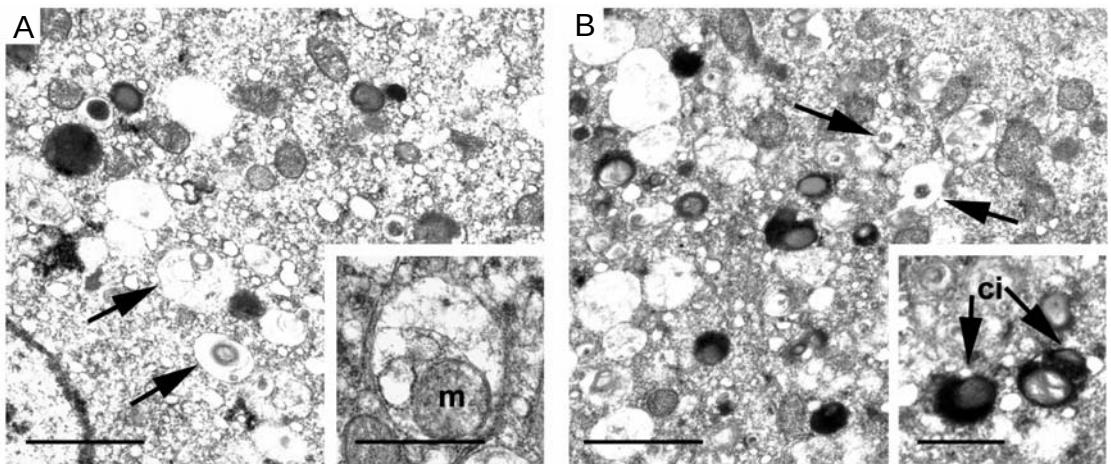


Figure 3.14 Transmission EM images of midgut sections from WT and *dronc*^{d5} white prepupal animals.

Arrows indicate small vesicles containing cytoplasmic contents. The inset in A shows an engulfed mitochondria (m) present only in WT midguts while the inset in B shows crystalline inclusion (ci) bodies common to both WT and *dronc*^{d5} midgut cells. Scale bars in A, A inset and B are 1 μm; B inset 0.5 μm.

BR-C, *E93* and *E74A* mutant midguts have distinct autophagic phenotypes and impact core cell death gene transcription differently, suggesting a complex relationship exists between apoptosis and autophagy for the correct removal of this tissue. Although *E93* mutants impact on *dronc* transcription, removal of *dronc* does not emulate the *E93* mutant phenotype as no obvious persistence of the larval lumen at 12 h RPF in $dronc^{d5}$ animals was observed. This suggests that while some aspects of autophagy are aberrant in both *E93* and *dronc* mutants, other unknown molecules must contribute to autophagic changes in *E93* mutants other than DRONC.

3.12 DRONC is required for salivary gland removal

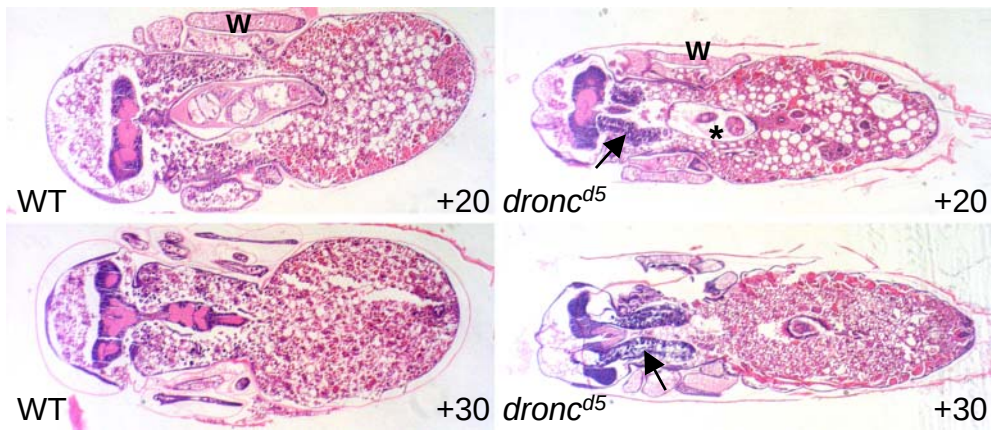
3.12.1 *dronc* mutant animals have persistent salivary glands

As the E93 and BR-C ecdysone-responsive transcription factor mutants disrupt core cell death gene transcription in salivary glands, including *dronc*, and also prevent their correct removal, *dronc* mutants are therefore likely to have aberrant salivary gland destruction. Evidence was recently provided using a *dronc* dominant-negative overexpression strategy and demonstrated a requirement of WT DRONC for DNA fragmentation, caspase activation, and general salivary gland removal (Martin and Baehrecke, 2004). Also, section 3.2.2 of this thesis outlines aberrant salivary gland removal in *KGO2994* animals, which have delayed salivary gland specific *dronc* expression and protein.

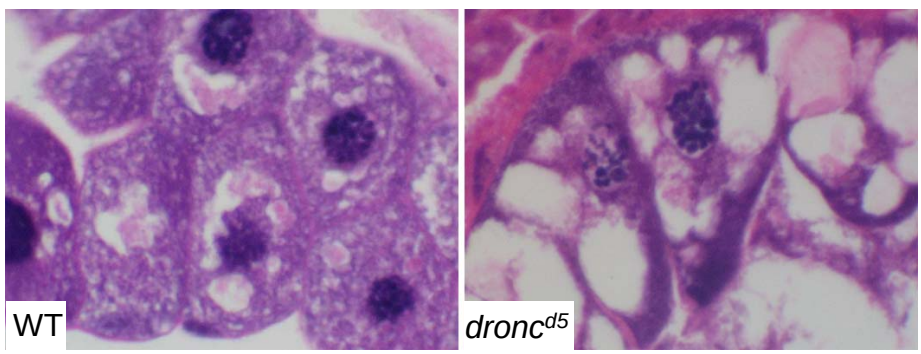
Figure 3.15 *dronc*^{d5} larval salivary glands persist with defects in vacuolar dynamics.

(A) WT and *dronc*^{d5} mutant animals were staged to the indicated times (+20 and +30 h RPF), fixed, sectioned and stained for histological analysis. Larval salivary glands persist beyond the WT time of salivary gland destruction in *dronc*^{d5} animals. Note adult wing (w) and eye (e) structures forming in *dronc*^{d5} and WT animals. Asterix indicates adult gut lumen with condensed residual larval midgut. All sections are identical magnification. **(B)** H & E stained sections of +13 h RPF salivary glands from WT and *dronc*^{d5} animals. More large eosin-negative vacuoles are present in *dronc*^{d5} salivary glands compared to WT controls.

A



B



To further assess the role of DRONC in development, *dronc*^{d5} and control animals were staged to 20 and 30 h RPF, fixed, paraffin embedded, sectioned and stained with haematoxylin and eosin. *dronc* mutant animals contained persistent salivary glands of an appearance similar to pre-histolysed controls including a visible duct surrounded by intact cells, invariably cuboidal with large nuclei (figure 3.15 A). WT animals of the same stage have lost their salivary glands. The formation of adult structures in *dronc*^{d5} animals, as occurs in the control, indicates continuing developmental progression and precludes the possibility that *dronc* mutant salivary glands persist due to arrested development at a stage prior to commencement of the salivary gland death programme. Intact glands were still present and of typical appearance at 30 h RPF (figure 3.15 A) and adult structures were present as in controls indicating continuing pupal development.

Autophagy in salivary glands is typified by vacuolar dynamics involving a shift from larger eosin-negative vacuoles to predominantly smaller eosin-positive vacuoles and occurs in the three hours prior to histolysis (Lee and Baehrecke, 2001; Lee et al., 2002b). Progression of autophagy, judged by vacuolar size prior to histolysis (13 h RPF), shows WT salivary gland cells containing variable sized eosin-positive vacuoles while *dronc*^{d5} cells primarily contain larger vacuoles typical of earlier developmental stages (figure 3.15 B). This indicates a failure of *dronc* mutant salivary glands, at these early stages of autophagy, to progress to a stage closer to the event of histolysis. This phenotype links aspects of the process of autophagy with the apoptotic core cell death machinery and specifically with DRONC function.

3.12.2 *dronc* mutant salivary glands are deficient for TUNEL

To investigate if DRONC is required for DNA fragmentation, salivary glands were dissected from *dronc*^{d5} and control animals staged prior to histolysis, previously shown to be a time when WT glands have TUNEL staining. Glands were fixed and TUNEL/Hoechst stained for confocal analysis. All *dronc*^{d5} animals dissected had intact salivary glands beyond the normal time of gland destruction with no TUNEL staining (figure 3.16). Hoechst staining of persistent glands shows that the cellular complement is maintained in mutant salivary glands as well as DNA integrity (figure 3.16 D and B inset).

3.12.3 Effector caspase activation is DRONC-dependent in salivary glands

If salivary gland removal is a *dronc*-dependent process, *dronc*^{d5} glands should be deficient in caspase activity. Western analysis showed no DRONC protein in the *dronc*^{d5} salivary glands at a time when WT glands have abundant DRONC (figure 3.17 A). Control and *dronc* mutant salivary gland homogenates were incubated with caspase-specific fluorogenic peptides to investigate caspase activity levels in salivary gland mutants prior to the normal time of histolysis. Unlike the observations in midguts deficient for DRONC, caspase activity was reduced in *dronc*^{d5} salivary glands at 14 h RPF compared to WT (figure 3.17 B).

Due to the promiscuity of caspase molecules on substrate peptides, caspase-3/DRICE-specific antibodies were employed to see if the reduction in VDVAD cleavage in *dronc*^{d5} salivary glands was due to reduced DRICE activation. Salivary glands from *dronc*^{d5} and WT animals at 14 h RPF were stained for active DRICE protein previously

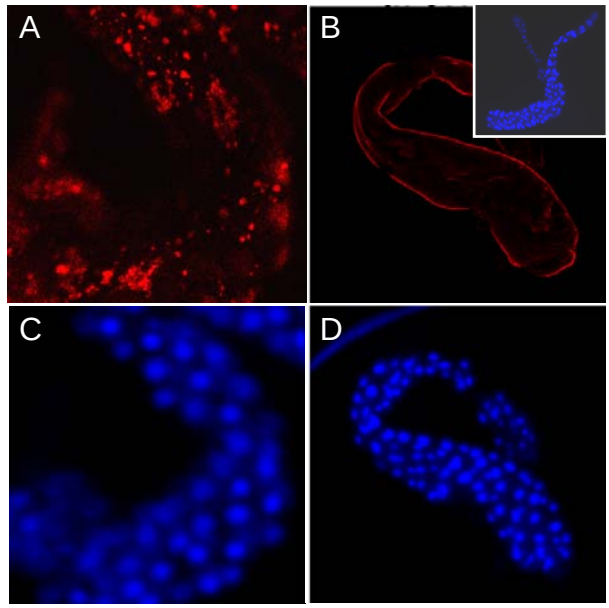


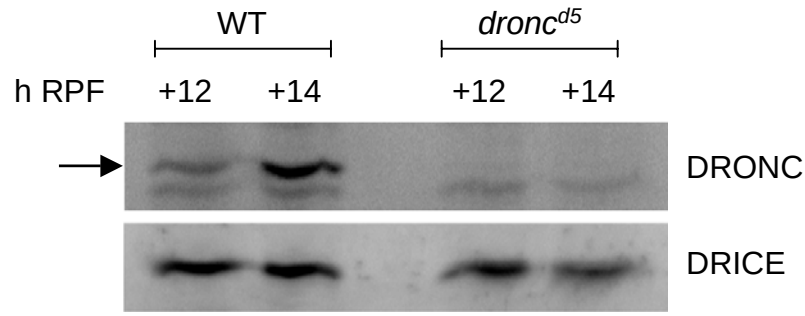
Figure 3.16 *dronc* mutant salivary gland TUNEL.

No TUNEL staining was observed in *dronc*^{d5} salivary glands from animals staged to +14 h RPF (B) unlike that observed in similarly staged control salivary glands (A). Hoechst staining demonstrates maintenance of *dronc*^{d5} structural integrity up to 48 h RPF (B inset). C and D shows Hoechst staining of control and *dronc*^{d5} salivary glands respectively.

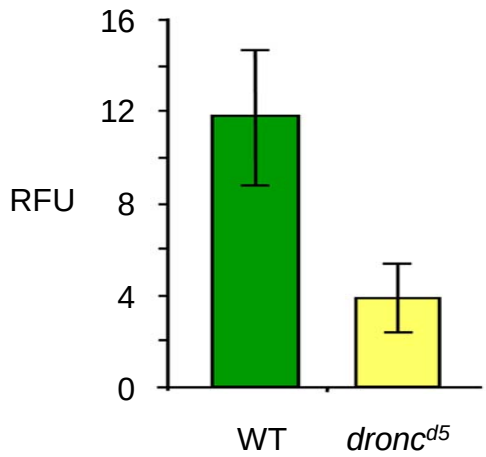
Figure 3.17 Caspase activity in persistent *dronc*^{d5} salivary glands.

(A) DRONC is absent from *dronc*^{d5} salivary glands whereas DRICE levels are unaffected. DRONC and DRICE were detected by immunoblotting. **(B)** Caspase activity is reduced in *dronc*^{d5} salivary glands at +14 h RPF. Caspase activity was measured on VDVAD-AMC substrate and expressed as relative fluorescence units (RFU). Values represent the mean ± SD derived from 3 independent experiments. **(C)** Reduced active DRICE staining in *dronc*^{d5} salivary glands at +14 h RPF compared to WT. In control experiments, no background staining was seen with the secondary antibodies alone.

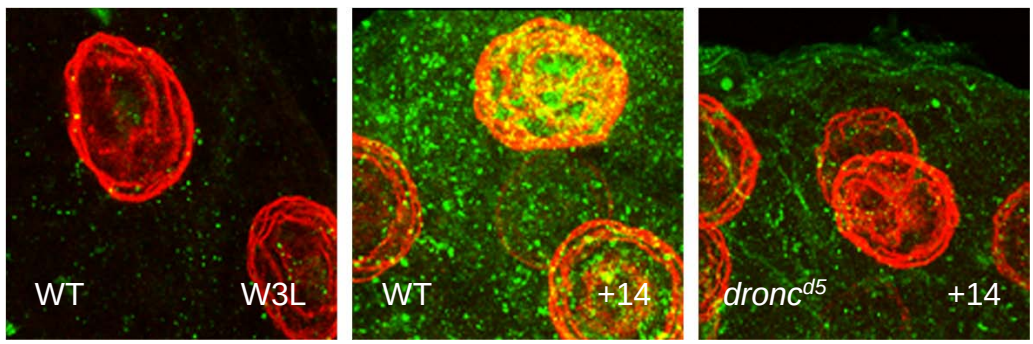
A


WT
dronc^d5
h RPF
+12
+14
+12
+14
DRONC
DRICE

B


16
12
RFU
8
4
0
WT
dronc^d5

C


WT
W3L
WT
+14
dronc^d5
+14

shown to be present in salivary glands at this stage (Martin and Baehrecke, 2004). An increase in active DRICE immunostaining in WT salivary glands at 14 h RPF was observed (figure 3.17 C), however, active DRICE immunostaining in *dronc*^{d5} salivary glands at 14 h RPF was significantly lower (figure 3.17 C) even though the levels of DRICE protein in *dronc*^{d5} and WT salivary glands at this stage were comparable (figure 3.17 A). These results indicate that effector caspase activation in salivary glands is DRONC-dependent, in contrast to the DRONC-independent caspase activity occurring in the larval midgut.

3.12.4 *dronc* mutant salivary gland cells have ultrastructural anomalies

Similarly staged WT and *dronc* mutant salivary glands were processed for SEM analysis for ultrastructural comparative analysis. WT salivary gland cells contained typical autophagic structures such as cytoplasmic contents within double-membrane vesicles and remnant organelles within vacuoles (figure 3.18 A and B). Other structures were also observed such as swirling membranous formations, not seen in the *dronc* mutant glands, and numerous small clear vesicles as previously reported at this stage (figure 3.18 A and B) (Lee and Baehrecke, 2001). Ultrastructural analysis of *dronc*^{d5} salivary glands at 14 h RPF showed a paucity of membrane bound autophagic bodies as observed in WT salivary glands and, as well as intact mitochondria, mitochondria that appeared ruptured (figure 3.18 C and D). Some 14 h RPF *dronc*^{d5} salivary gland cells still had large vacuoles containing fibre-like structures as well as much smaller vesicles with unidentified contents (figure 3.18 D), all of which indicates that aberrant autophagy occurs in *dronc*^{d5} salivary glands.

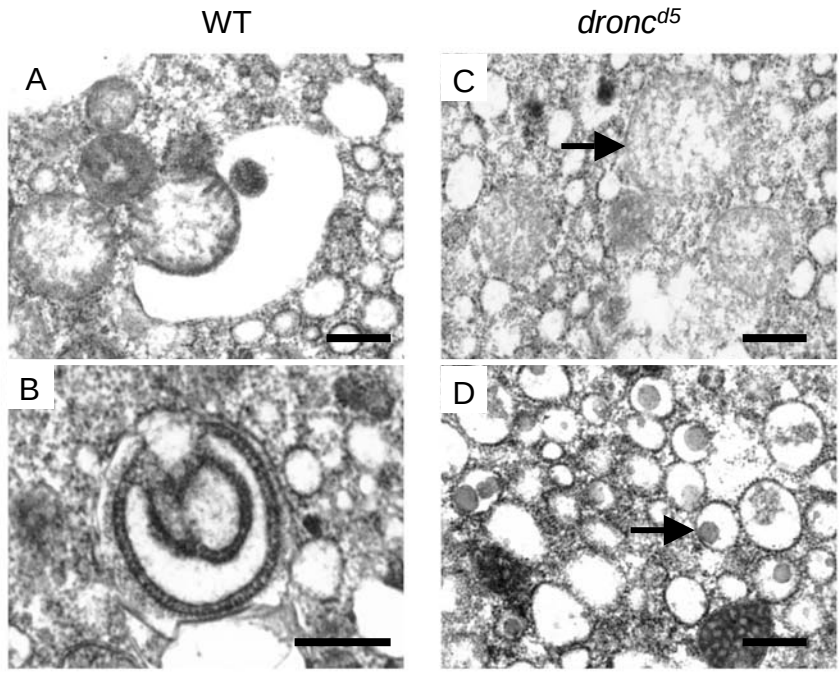


Figure 3.18 Transmission EM images of +14 h RPF WT and *dronc*^{d5} salivary glands.

Fields show cytoplasmic changes in the WT **(A and B)** and *dronc*^{d5} **(C and D)** salivary gland cells. A and B show membrane bound autophagic structures observed in WT histolysing salivary gland cells. In B, a double membrane bound autophagic body is shown. In C and D, while much smaller vacuolised cytoplasmic structures are present in some cells (arrow in D), larger membrane bound autophagic structures were not seen. The arrow in C indicates a fragmenting mitochondrion. All scale bars represent 0.5μm.

Total protein extracted from WT and *dronc*^{d5} was subjected to Western blotting to assess whether Lamin is cleaved in the absence of DRONC. Lamin appears to be cleaved prior to salivary gland histolysis to a similar extent in both *dronc*^{d5} and control animals (figure 3.19). This is in contrast to recent data from Martin and Baehrecke (2004) who demonstrate that, although nominal disruption of Lamin cleavage occurs in E93 and is prevented in BR-C mutant salivary glands, p35 and dominant negative DRONC expression reduce Lamin cleavage.

3.12.5 The PCD genetic regulatory pathway is active in *dronc*^{d5} salivary glands

To assess whether the genetic regulatory hierarchy preceding salivary gland removal is active in *dronc*^{d5} animals, cell death gene expression in *dronc*^{d5} and control salivary glands was analysed by RT-PCR. Figure 3.20 shows that a key cell death regulator, the transcription factor E93 (which is required for *dronc* transcription in the salivary glands), is upregulated in *dronc*^{d5} salivary glands at a time similar to controls. *hid* transcription was also observed, although at levels lower than WT (figure 3.20), indicating that components of the cell death genetic hierarchy are initiated in the *dronc* mutant salivary glands at relevant developmental times. This indicates that transduction of the cell death transcriptional cascade is gated by the absence of DRONC in *dronc*^{d5} salivary glands and precludes the possibility that developmental arrest or retardation of developmental progression is responsible for persistence of the *dronc*^{d5} salivary glands.

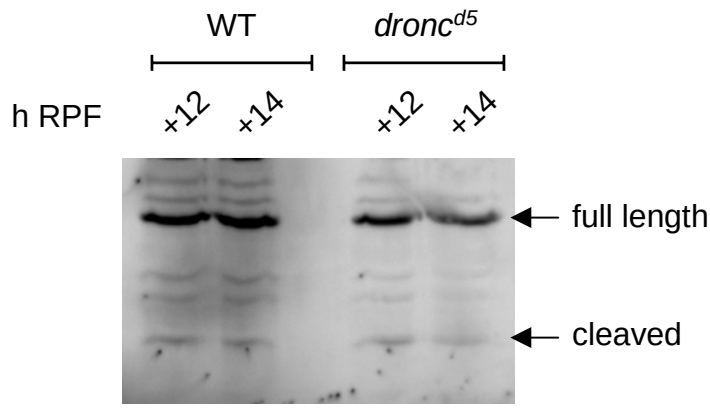


Figure 3.19 Lamin cleavage occurs in *dronc^{d5}* salivary glands.

Protein was extracted from WT and *dronc^{d5}* salivary glands from animals staged to the indicated times and blotted for Lamin cleavage status. The 45 kD cleavage product is present in both mutant and control lanes indicating that Lamin is targeted and cleaved during salivary gland histolysis in the absence of DRONC.

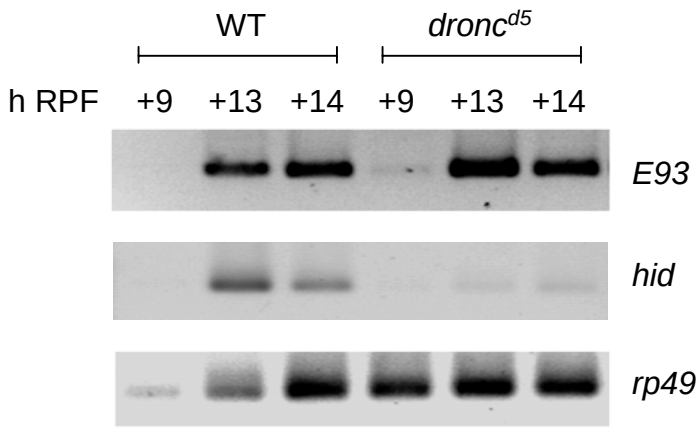


Figure 3.20 The PCD pathway is active in *dronc^{d5}* salivary glands.

RT-PCR showing that *E93* and *hid* are expressed in *dronc^{d5}* salivary glands prior to the time of normal salivary gland histolysis. *rp49* was used as a loading control.

3.13 DRONC is required for RPR-, HID- and GRIM-induced cell death in the eye

As DRONC has been shown to function in the RPR, HID and GRIM cell death pathway (Meier et al., 2000; Quinn et al., 2000), halving the dosage of DRONC should reduce the phenotype resulting from RPR/HID/GRIM overexpression. By exploiting the GAL4-UAS system to overexpress *rpr*, *hid* or *grim* in the larval eye disc to cause ectopic cell death resulting in an ablated eye phenotype, loss of one copy of *dronc* in these overexpression genetic backgrounds, achieved by crossing these flies with the *dronc*^{d5} animals (including the complementation transgene), resulted in suppression of the respective eye phenotypes (figure 3.21). This results from the death-signalling pathway, induced by H99 gene overexpression, failing to be transduced to effector caspases by DRONC.

3.14 *dronc* is required for radiation-induced cell death

To investigate the role of DRONC in the DNA damage-induced apoptotic pathway, *dronc*^{d5} and control third instar larvae were treated with γ radiation and analysed for effects on cell death or caspase activity. After treatment with 8 Gy γ-radiation, WT larvae had significant increases in eye disc cell death as observed by AO staining (figure 3.22 A). No increase in AO staining was observed in *dronc*^{d5} eye discs following irradiation (figure 3.22 A). Whole animal lysates from the same experiment showed an increase in caspase activity, as determined by VDVAD-, DEVD- and VEID-AMC cleavage following radiation exposure of control larvae (figure 3.21 B). Consistent with the lack of AO staining following irradiation, no significant increase in VDVAD or VEID cleavage was observed in *dronc*^{d5} animals following irradiation (figure 3.22 B).

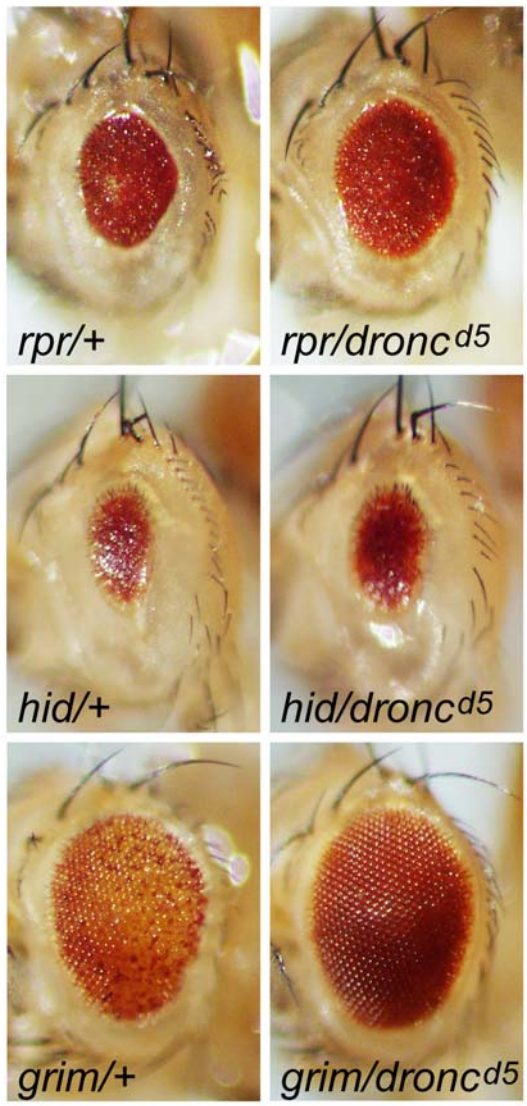


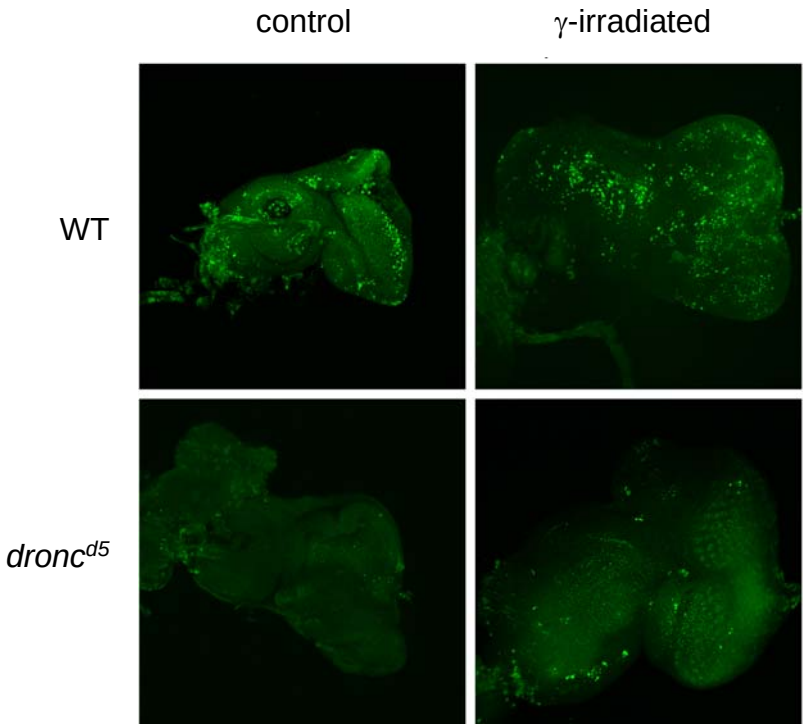
Figure 3.21 DRONC functions in the RPR, HID and GRIM induced cell death pathway.

GMR-rpr/+, *GMR-hid/+* and *GMR-grim/+* eye phenotypes are all reduced in severity by halving the dose of DRONC (*GMR-rpr/+; dronc^{d5}/+, GMR-hid/+; dronc^{d5}/+ and GMR-grim/+; dronc^{d5}/+*, respectively). Genetic interaction crosses were performed at 25℃.

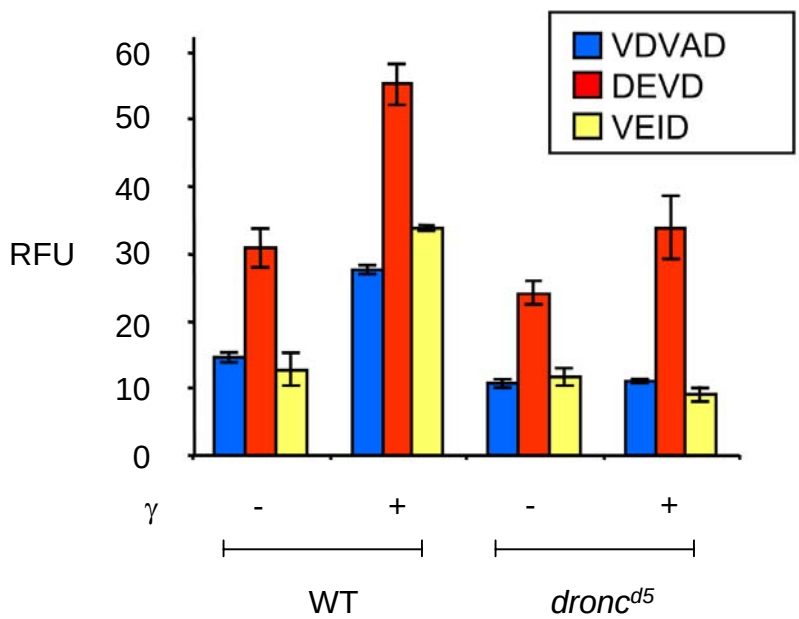
Figure 3.22 DRONC is required for stress-induced PCD and caspase activation *in vivo*.

(A) AO staining of WT and *dronc*^{d5} third instar larval eye discs treated with, or without, 8 Gy γ-radiation. No increase in AO staining in response to irradiation is observed in *dronc*^{d5} eye discs compared to WT. Note untreated *dronc*^{d5} eye discs have little or no AO positive cells compared to untreated WT eye discs. **(B)** Caspase activity in whole WT and *dronc*^{d5} larvae. No increase was observed in caspase activity for VDVAD and VEID substrates in *dronc*^{d5} animals following γ-irradiation. A marginal increase in *dronc*^{d5} DEVDase activity was seen. The error bars represent mean ± SEM for data derived from three experiments.

A


control
γ-irradiated
WT
dronc^d5

B


VDVAD
DEVD
VEID
RFU
60
50
40
30
20
10
0
γ
-
+
-
+
WT
dronc^d5

Although DEVD showed a marginal increase in activity it was less than half the control increase.

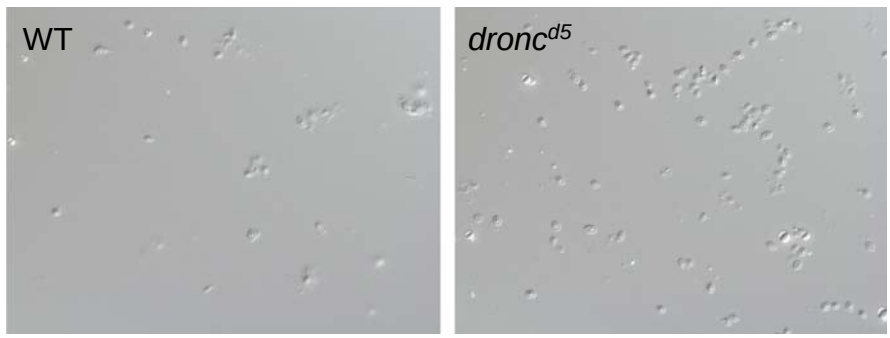
3.15 *dronc*^{d5} animals have increased blood cells

Wandering third instar *dronc*^{d5} larvae were assessed for abnormalities in blood cell development. Haemolymph from *dronc*^{d5} and WT animals was extracted and cell fields photographed and cell numbers counted. WT animals at the late larval stages have very few haemocyte cells whereas *dronc*^{d5} animals have high blood cell numbers (figure 3.23 A and B). Whether this is due to increased cell proliferation or reduced cell death has not been determined, however, this data is consistent with Chew et al (2004) who also observed increased haemocytes in *dronc* mutant animals. *Ex vivo* culture of blood cells from this mutant demonstrated they were refractory to multiple cell death stimuli, however hormone induced cell death was not tested.

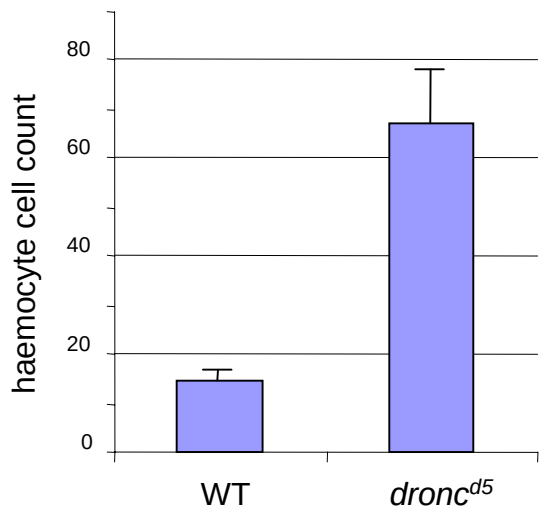
Figure 3.23 *dronc* mutants have persistent haemocytes.

(A) *dronc*^{d5} and WT wandering third instar larvae were collected and the haemolymph extracted for analysis. 2 μl of haemolymph was diluted 1:2 with PBS, mounted on a glass microscopy slide and photographed with a digital camera. *dronc*^{d5} mutants had significantly greater blood cell numbers compared to control animals. **(B)** Cell fields were counted and graphed to demonstrate the empirically significant difference in haemocyte numbers. WT and *dronc*^{d5} animals had an average blood cell number in a 40 X mag field of 14.25 (± 2.22) and 67.50 (± 10.85) respectively. The error bars represent mean ± SEM.

A



B



3.16 Discussion

Analysis of the *dronc* mutant demonstrates that DRONC is required for cell deaths in specific pathways. For example, DRONC was required for developmental cell death in a variety of postembryonic tissues, apoptosis resulting from radiation-induced DNA damage, and the removal of the larval salivary glands during metamorphosis. A lack of DRONC however had no effect on the timing or extent of larval midgut histolysis, indicating DRONC is not required for midgut cell death.

The mutant is a specific point mutation in the *dronc* gene replacing a partially deleted allele. The flanking gene, *CG6685,* was shown to be essential for *Drosophila* development and *dronc* deletion may effect *CG6685* regulation by removal of essential promoter elements. Consequently, the *dronc* mutant described is an effective way of generating a *dronc*-specific mutant when using an imprecise P-element excision strategy.

Previous studies have implicated an essential role for caspases in aspects of midgut removal, however, until now DRONC redundancy in this tissue has not been demonstrated. As dying midguts from *dronc* null animals contained significant amounts of effector caspase activity, this indicates that DRONC is not required for downstream caspase activation in this tissue. *E93* mutants impact *dronc* transcription, however, removal of DRONC does not emulate the *E93* mutant midgut phenotype as no obvious persistence of the larval lumen at 12 h RPF in $dronc^{d5}$ animals was observed.

As *dronc* null animals from heterozygous parents have an apparently normal developmental schedule until the early pupal stage, maternal *dronc* must play a

significant role in achieving an advanced stage of development in these animals. As evidenced recently (Huh et al., 2004a), there may be compensatory mechanisms occurring preventing excess or insufficient cellular complements in response to cell death programs. The hypothesis that tissue growth may be linked to cell death through caspase function is discussed in chapter 6. The data suggests that in the absence of DRONC, pre-metamorphic development, including the temporal schedule, is insufficiently disrupted to affect puparium formation, and the observed deficits in larval cell death may only have consequences in early pupal development when the salivary gland destruction defect occurs. In respect to initiation of histolysis and cell death gene requirements and redundancies in the salivary glands and midguts, it is clear that core cell death machinery components are used differently. It is therefore not surprising to observe differential consequences of DRONC ablation between the midgut and salivary glands.

The *dronc* mutation described by Chew et al (2004) (*dronc*^{51}) was generated by P-element excision resulting in removal of *dronc* but not *CG6685 coding* sequence. As described, this would remove the regulatory sequences of *CG6685* and, since this gene is required for normal development, may result in a phenotype different from the *dronc*^{d5} mutant. *dronc*^{51} homozygotes are late larval lethal and have increased blood cells, however, very few animals pupated and thus assessment of salivary gland and midgut destruction was not possible. Furthermore, the CNS structures and imaginal discs were enlarged in some *dronc*^{51} animals, possibly a result of reduced cell death in these tissues. The fact that *dronc*^{d5} animals had no similar phenotype indicates compensatory mechanisms may exist to maintain organ

size in the absence of DRONC. The complementation transgenes described in this thesis were supplied to Chew and colleagues to test their ability to rescue the *dronc*^{51} lethal phenotype. The transgene containing functional *dronc* sequence rescued *dronc*^{51} lethality but with very low penetrance. No data has been received regarding replacing CG6685 function in *dronc*^{51} animals.

The following unexpected findings were revealed by analysis of the *dronc*^{d5} mutant; (i) DRONC is essential for *Drosophila* development, (ii) although DRONC is the only CARD-containing *Drosophila* caspase, DRONC-independent caspase activation can occur, and (iii) midgut destruction can still occur in the complete absence of DRONC. As DRONC has recently been implicated in some non-apoptotic events (Geisbrecht and Montell, 2004; Huh et al., 2004a; Huh et al., 2004b), generation of a *dronc*-specific mutant now makes the exploration of these alternative roles possible.

Chapter 4

Distinct promoter regions regulate spatial and temporal expression of the Drosophila caspase dronc

4.1 Introduction

Components of the cell death pathway, including caspases, are present in normal cells, and thus it has been generally accepted that PCD is primarily regulated post-transcriptionally. However, many components of the core death machinery, including some caspases, are transcriptionally regulated during cell death signalling suggesting anti-death and -survival factor levels in the cell may be crucial for controlling the apoptotic program (Baehrecke, 2002). Signals such as hormones and growth factors can regulate the death program by controlling the balance between these core cell death machinery molecules (Baehrecke, 2002). Therefore to further understand cell death regulation, it is essential to understand the hormone-mediated transcriptional control of apoptosis.

Ecdysone pulses throughout fly development regulates moulting, cell proliferation, differentiation and PCD (Baehrecke, 2002; Truman and Riddiford, 2002). Regulation of PCD by ecdysone is complex and involves EcR/Usp and a number of induced transcription factors including *βFTZ-F1, BR-C, E74, E75* and *E93* (Baehrecke, 2000; Baehrecke, 2002; Broadus et al., 1999; DiBello et al., 1991; Fletcher et al., 1995; Jiang et al., 2000; Karim et al., 1993; Lee and Baehrecke, 2001; Lee et al., 2000). The combinatorial and tissue-specific distribution of these factors regulate the expression of the cell death genes *rpr*, *hid*, *dark*, *dronc*, and the death inhibitors *diap1* and *diap2* (Baehrecke, 2000; Baehrecke, 2002). DRONC was the first caspase shown to be regulated by a steroid hormone (Dorstyn et al., 1999a) and the importance of *dronc* regulation in ecdysone-mediated cell death was established in chapter 3. The molecular mechanisms of *dronc* regulation however remain poorly understood.

The aim of this chapter was to identify regions of the *dronc* promoter involved in controlling its restricted temporal and spatial expression pattern. This was achieved by employing a transgenic *LacZ* promoter-reporter system in combination with mutants of the early response ecdysone transcription factors *BR-C*, *E93* and *E74*. Specific regions of the *dronc* promoter are shown to be involved in ecdysone-dependent and -independent temporal and spatial *dronc* regulation during *Drosophila* development. The minimal region of *dronc* promoter required to emulate endogenous expression in midgut and salivary glands was 2.8 kb and was also required for correct BR-C- and E93-mediated reporter expression.

Results

4.2 *dronc* promoter-driven *LacZ* expression in embryos and adult ovaries

Previous data show that *dronc* transcript is present at high levels in embryos, at very low levels through the first and second larval instars, and increases dramatically at pupariation (Dorstyn et al., 1999a). High levels are observed in midguts and salivary glands prior to their destruction while low levels of *dronc* transcript is seen in the larval eye discs and brain lobes, whereas strong expression is evident in adult egg chambers (Dorstyn et al., 1999a). To study the *in vivo* regulation of *dronc* expression, a number of transgenic constructs were generated containing various lengths of the *dronc* promoter sequences fused to a *LacZ* gene containing a nuclear localization signal (figure 4.1). Three reporter constructs were generated containing different promoter lengths; 0.54, 1.1 and 2.8 kb. Transgenic *Drosophila* lines containing one of each of these promoter-reporter constructs were generated by established techniques. To ensure that the chromosomal insertion location did not have any temporal and/or spatial regulatory influences or repressive effects on the expression of the reporter constructs, several transgenic lines per promoter construct were analysed.

Initial analysis involved standard X-gal staining of embryos of each of the reporter lines and a WT control. Analysis of βgal activity in early embryos collected at 0-4 h after egg laying (AEL) demonstrated that while 1.1 and 2.8 kb promoter-*LacZ* lines showed βgal expression, the 0.54 kb promoter-*LacZ* line and the WT did not (figure 4.2 A). The expression levels of the 1.1 and 2.8 kb have only low-level βgal staining at this time indicating low-level transcription or only limited translation of the

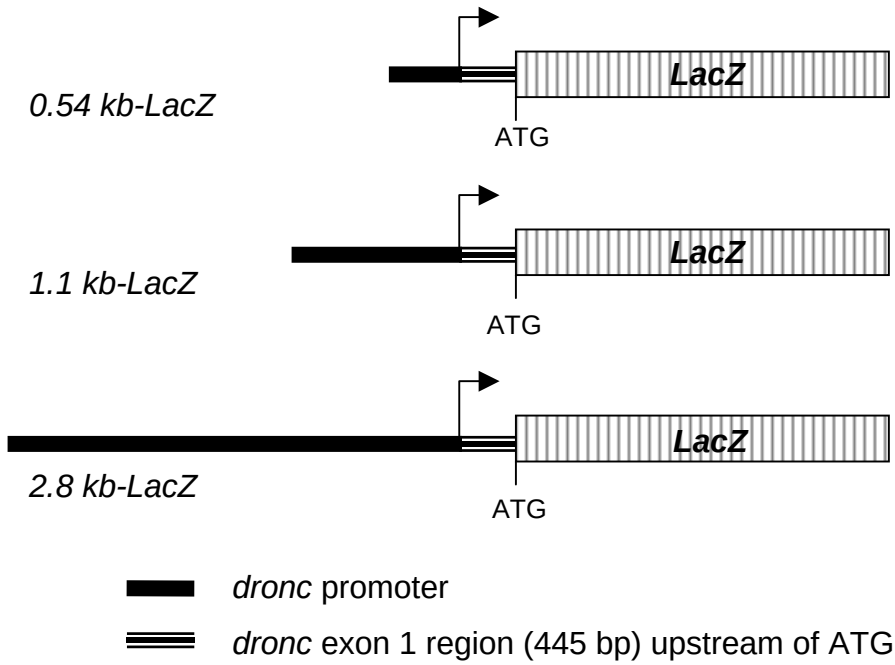
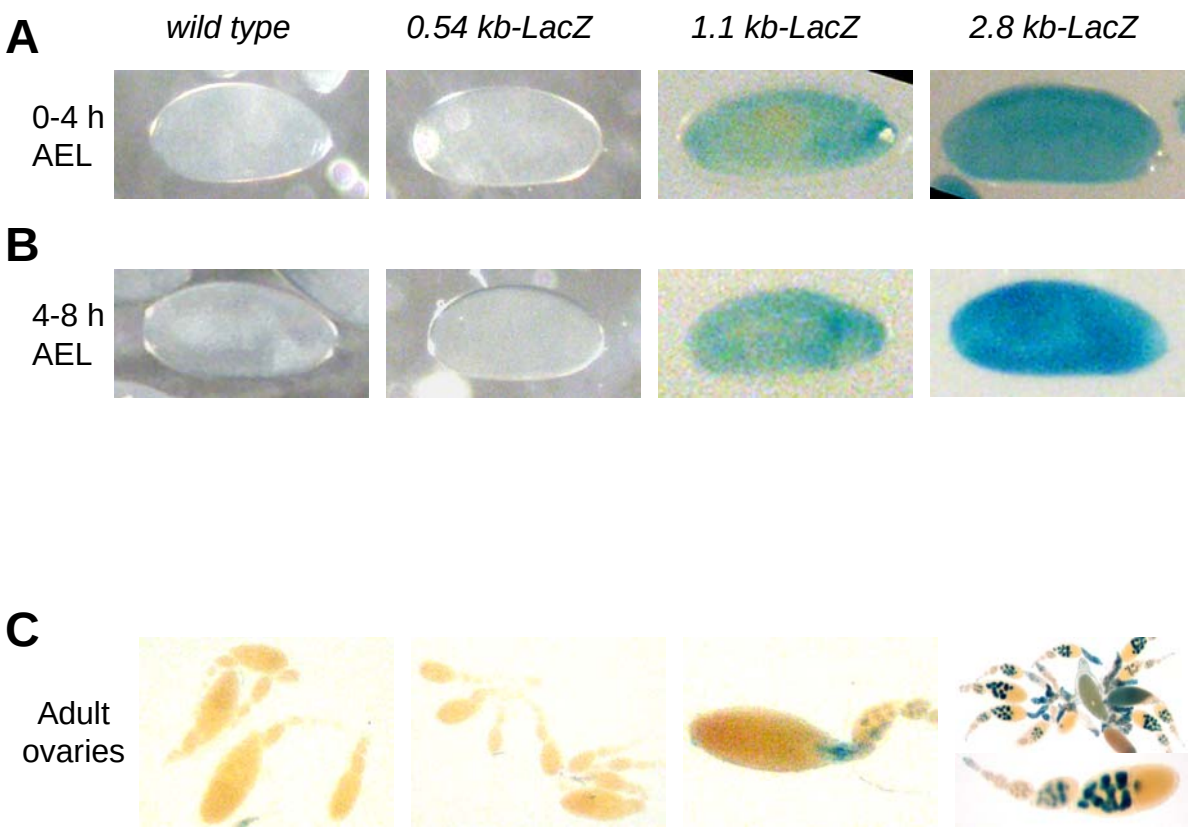


Figure 4.1 *dronc* promoter-*LacZ* constructs used to generate transgenic flies.

Various lengths of the promoter upstream of the *dronc* transcription start site (shown by an arrow) were cloned upstream of a *LacZ* reporter gene containing a nuclear localization signal into the pCaSpeR-4 *Drosophila* transformation vector. All constructs also contain the 445 bp noncoding region of the *dronc* exon 1. In all constructs the initiator ATG codon is the natural *dronc* initiation codon.

Figure 4.2 Expression of various *dronc* promoter-*LacZ* transgenes in WT and transgenic *Drosophila* embryos (*A*, *B*), egg chambers (*C*) and testis (D).

In *A* and *B*, time shown on the left represents hours after egg laying (h AEL). Dechorionated mixed stage embryos 0-4 h AEL (*A*) or 4-8 h AEL (*B*) or dissected ovaries (*C*) from adult flies were stained with X-gal as described in *Materials and Methods*. After mounting, the stained samples were photographed using an Olympus microscope fitted with a digital camera. βgal staining in transgenic flies containing the 1.1 and 2.8 kb promoter transgene appear diffused in embryos (*A*, *B*) and as distinct nuclear spots in the nurse cells in the egg chambers (*C*). In the wild type and 0.54 kb-promoter-*LacZ* embryos (*A*, *B*) no X-gal staining was apparent. Phase contrast microscopy was used to visualize these unstained embryos. All reporter lines are homozygous for the respective reporter transgenes. Multiple lines derived from each transgenic construct were analysed for βgal activity. The embryos and ovarioles are representative examples of transgene expression. (D) Adult testis from *2.8kb-nLacZ* animals have βgal activity throughout the tissue.


A
wild type
0.54 kb-LacZ
1.1 kb-LacZ
2.8 kb-LacZ
0-4 h AEL
B
4-8 h AEL
C
Adult ovaries
D
Adult 2.8kb testis

maternally deposited transcript occurring in embryos in the initial 4 h AEL. When embryos were aged for another 4 h AEL (4-8 h AEL), only the 2.8 kb reporter appeared to increase in activity, indicated by a significant increase in βgal staining (figure 4.2 B). This suggests that while 1.1 kb of *dronc* promoter may be driving the basal expression of the reporter, 2.8 kb is required for maximal promoter activity upon commencement of zygotic gene expression. It was previously shown that *dronc* transcript is maternally deposited in the early embryos (Dorstyn et al., 1999a). Zygotic expression of genes in *Drosophila* does not begin until around stage 5 (~3 h AEL), therefore, βgal expression seen in embryos 0-4 h AEL may largely represent maternally deposited *LacZ*.

To gauge whether the reporter activity in early embryos was due to maternal contributions, the level of βgal in egg chambers of adult ovaries from transgenic lines was tested. Oocytes are provided with nutrients, protein and message RNA by 15 nurse cells, all of which are surrounded by somatically-derived follicle cells (Foley and Cooley, 1998; McCall and Steller, 1998; Spradling, 1993). Correct development of the *Drosophila* egg chamber requires a mid oogenesis stress-induced cell death checkpoint and a late oogenesis death program occurring in the 15 nurse cells following transfer of their cytoplasm to the oocyte (Chao and Nagoshi, 1999; Foley and Cooley, 1998; McCall and Steller, 1998; Nezis et al., 2000). Active DRICE is associated primarily with the stress-induced cell death at mid oogenesis (Peterson et al., 2003). Ovaries were dissected from adult animals and stained for βgal activity. As observed in embryos, no βgal expression was evident in ovariole egg chambers or nurse cells from lines carrying the 0.54 kb-LacZ construct (figure 4.2 C). However,

both 1.1 and 2.8 kb promoters were able to drive *LacZ* expression in nurse cells (figure 4.2 C), suggesting that the 1.1 kb promoter was sufficient for βgal expression in the developing follicle. The staining shows high activity of the 2.8 kb reporter compared to the 1.1 kb line, which seems, as in the embryos, to have basal or low level activity. As nurse cells dump their contents into the developing oocyte, the βgal activity in 0-4 h embryos with 1.1 kb and 2.8 kb promoter transgenes appear to primarily be due to the maternally deposited transcript. As the activity of the 2.8 kb-*LacZ* transgene is higher in 4-8 h embryos, it can be concluded that 2.8 kb of *dronc* promoter is sufficient to direct the limited zygotic expression of *dronc* in embryos.

Adult 2.8 kb-*LacZ* animals had significant βgal activity in the testis (figure 4.2 D). DRONC has been shown to be required for correct spermatid individualisation, but interestingly, not for the activation of DRICE in the testis (Huh et al., 2004b).

4.3 *dronc* regulation in midguts and salivary glands during metamorphosis

dronc is tightly regulated in the larval salivary glands and midguts during metamorphosis. To assess whether the transgenes contained sufficient regulatory elements to control tissue-specific expression emulating endogenous *dronc*, the larval midguts were assayed by X-gal staining and quantitative βgal analysis. As a major ecdysone-mediated upregulation of *dronc* expression occurs in the midgut at the larval-pupal transition, expression of the promoter-*LacZ* transgenes was analysed before and after this developmental progression. In midguts dissected from late third instar larvae (-12 h RPF), both 1.1 and 2.8 kb of *dronc* promoter was able to direct *LacZ* expression, however, the 1.1 kb appeared to have greater intensity relative to

the 2.8 kb staining. Furthermore, while both appear to have quite low-level X-gal staining, the qualitative aspects or distribution of staining is markedly different between the transgenes, with only 1.1 kb-*LacZ* expressed in the gastric caecae (Figure 4.3 A). In response to the late larval ecdysone pulse both transgenes increase transcriptional activity, however, the 1.1 kb construct has greater staining distribution and intensity compared to the 2.8 kb early prepupal midguts (+1 h RPF) which still lacks gastric caecae staining (Figure 4.3 A). Both constructs have intense staining throughout the posterior midgut.

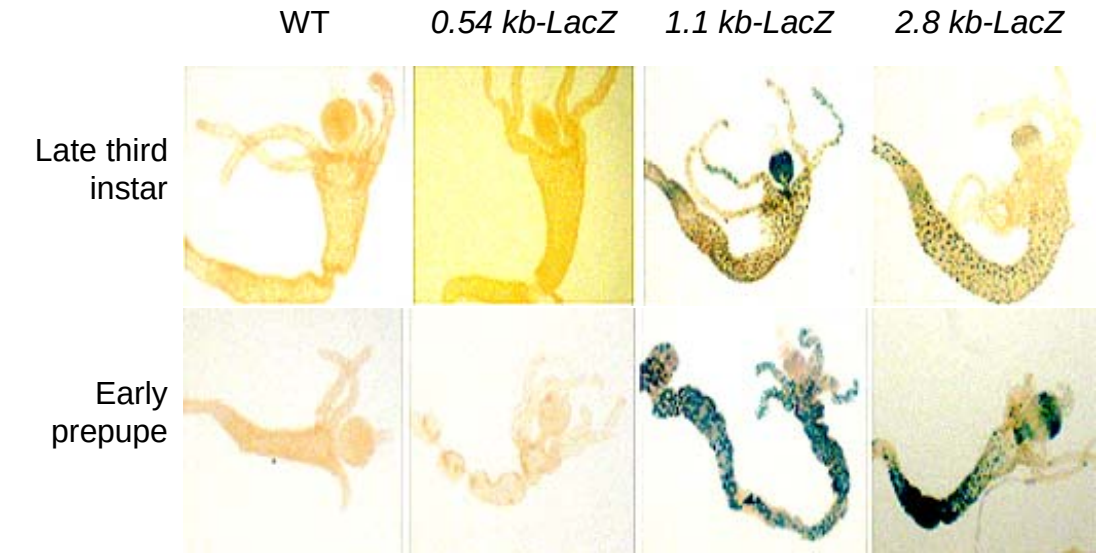
These observations indicate that the different spatial expression profiles of the two promoter constructs may be due to the heterogeneity of this tissue. Midguts were dissected and homogenized for βgal activity assays from similarly staged 1.1 kb and 2.8 kb-*LacZ* animals. In support of the X-gal staining data, both transgenes respond to the late larval ecdysone pulse by demonstrating an increase in activity in early prepupal (+1 h RPF) midguts relative to late larval midguts (figure 4.3 B). The data demonstrated higher activity of the 1.1kb-*LacZ* transgene compared to the 2.8 kb-*LacZ* construct, i.e. higher pre-puparium formation levels than the 2.8 kb post-puparium formation levels. Activity levels of the two reporter constructs suggest the presence of a repressor element unique to the 2.8 kb region. 0.54 kb promoter was unable to drive reporter gene expression while the WT midguts had no detectable βgal activity (Figure 4.3 A and B).

To determine the ability of the reporter constructs to respond to the late prepupal ecdysone pulse rather than the late larval pulse in the salivary glands, animals were aged to the late larval (-12 h RPF), prepupal (+1 h RPF) and early pupal

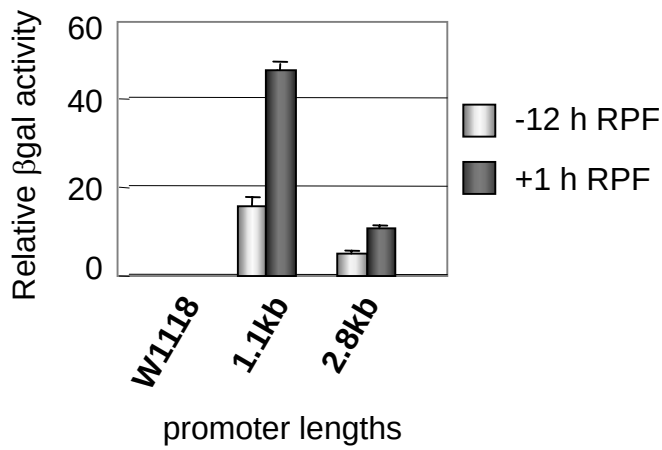
Figure 4.3 *dronc* promoter-reporter activity in the midgut.

(A) Expression of various *dronc* promoter-*LacZ* transgenes in the midguts of wild type and transgenic animals. Midguts were dissected from late third instar larvae (-12 h RPF) and early prepupae (+1 h RPF) and stained with X-gal. The prepupal 2.8 kb promoter-*LacZ* reporter expression pattern is similar to that observed in late third instar larvae. No βgal staining was observed in the midgut wild type controls or in 0.54 kb promoter-*LacZ* transgenic lines. **(B)** CPRG quantitative βgal assays. Promoter-reporter and control midguts were dissected from animals staged to the indicated times, homogenised and assayed for βgal activity. While both 1.1 kb and 2.8 kb transgenes increase activity following the late larval ecdysone pulse, the response by the 1.1kb is greater with higher basal levels. WT midguts have no detectable βgal activity.

A


WT
0.54 kb-LacZ
1.1 kb-LacZ
2.8 kb-LacZ
Late third instar
Early prepupe

B


Relative βgal activity
60
40
20
0
-12 h RPF
+1 h RPF
W1118
1.1kb
2.8kb
promoter lengths

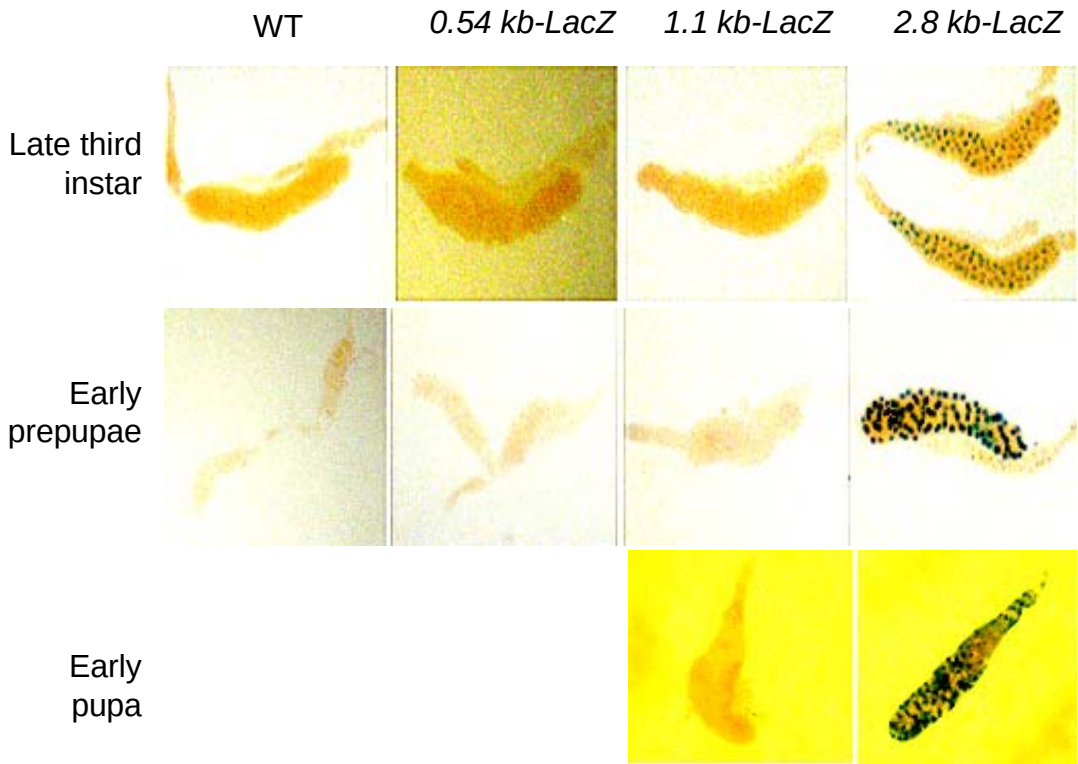
(+12 h RPF) stages, salivary glands dissected and stained for βgal activity. In salivary glands isolated from late third instar larvae or early prepupae, both WT and 0.54 kb promoter-*LacZ* transgenes failed to express, however, the 2.8 kb promoter-*LacZ* was expressed (Figure 4.4, upper two panels). The 1.1 kb-*LacZ* transgene had no evident staining at any stage, however, very low level staining could be observed if prolonged incubations in X-gal were performed. As salivary glands commence histolysis ~13 h later than the midgut, expression of the 2.8 kb-*LacZ* transgene in salivary glands isolated from early pupae (+12 h RPF) was analysed. Only 2.8 kb promoter was able to drive reporter expression in salivary glands at this stage of development (Figure 4.4 A). Quantitative analysis of the 2.8 kb-*LacZ* salivary gland expression profile demonstrated transcriptional activity at both the late larval and late prepupal ecdysone peaks (figure 4.4 B). While a small increase is observed at the larval-prepupal transition, the most marked increase is observed over the prepupal-pupal boundary, consistent with the transcriptional profile of endogenous *dronc*. This suggests sufficient regulatory information resides in the 2.8 kb promoter sequence to direct correct salivary gland specific *dronc* expression at a developmentally appropriate time.

To directly compare the 2.8 kb-*LacZ* transcriptional profiles in the midgut and salivary glands with the endogenous *dronc* transcript, RNA was prepared from these two tissues for Northern blotting. Midgut RNA was collected from third instar animals (-12 h RPF) and from early prepupal (+1 h RPF) animals, while salivary gland RNA was collected from animals staged to the time just prior to the time of salivary gland destruction (+12 h RPF). Reporter transcription was observed to emulate endogenous

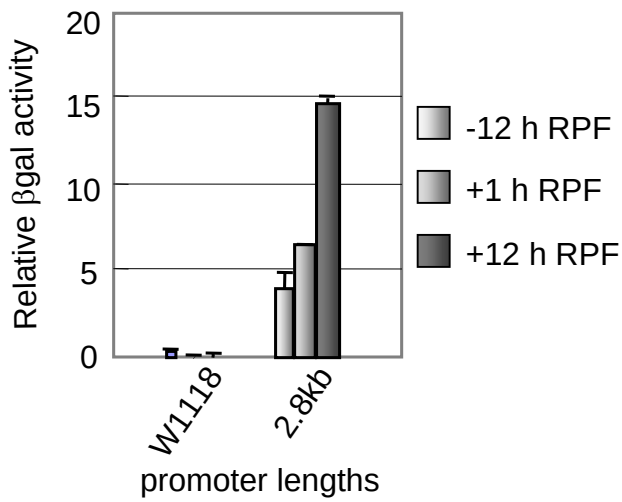
Figure 4.4 Expression of *dronc* promoter-*LacZ* transgenes in the salivary glands of wild type and transgenic animals.

(A) Salivary glands were dissected from late third instar larvae (-12 h RPF), early prepupae (+1 h RPF), and early prepupal stages (+12 h RPF) and stained with X-gal. No βgal staining was observed in the salivary glands from wild type controls or in 0.54 kb promoter-*LacZ* transgenic lines. Only very low staining was ever observed in 1.1 kb-*LacZ* salivary glands and only after extended incubation periods in X-gal. **(B)** CPRG quantitative βgal assays. 2.8kb promoter-reporter and control salivary glands were dissected from animals staged to the indicated times, homogenised and assayed for βgal activity. 2.8 kb-*LacZ* activity increased dramatically in the salivary glands in response to the late prepupal ecdysone pulse indicating sufficient promoter elements are present to regulate *droncs* correct spatial/temporal expression profile.

A


WT
0.54 kb-LacZ
1.1 kb-LacZ
2.8 kb-LacZ
Late third instar
Early prepupae
Early pupa

B


Relative βgal activity
20
15
10
5
0
W1118
2.8kb
promoter lengths
-12 h RPF
+1 h RPF
+12 h RPF

dronc transcription in both midguts and salivary glands in response to the late larval and late prepupal ecdysone pulses respectively (figure 4.5). The low levels of βgal activity observed prior to these increases are most likely due to an accumulation of transgene product resulting from continued but low level basal transcription.

These results suggest that while the 1.1 kb promoter is sufficient to drive expression of *dronc* in the midgut, 2.8 kb of the promoter is required for driving optimal expression in salivary glands. Furthermore, the data indicate that a region between 1.1-2.8 kb of the promoter is essential for salivary gland-specific *dronc* expression.

4.4 Expression of the promoter-*LacZ* transgenes in larval brain lobes

To identify promoter regions required for *dronc* expression in other tissues, brain lobes were dissected from controls and promoter-reporter lines and stained with X-gal. Previous studies have shown that *dronc* is expressed in third instar larval brain lobes (Dorstyn et al., 1999a). Similar to that observed in egg chamber nurse cells, neither WT, 0.54 or 1.1 kb promoter was able to drive detectable reporter expression levels in brain lobes, while the 2.8 kb-*LacZ* transgene produced high levels of βgal activity (figure 4.6). This indicates that, as in the larval salivary glands, a region of promoter greater than 1.1 kb and unique to the 2.8 kb transgene region is required for expression in this tissue.

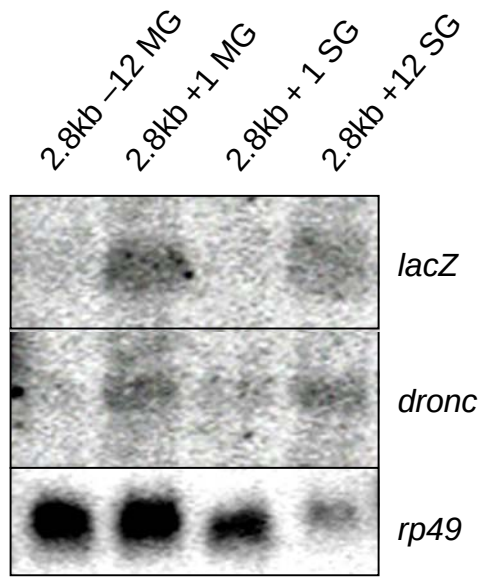


Figure 4.5 2.8kb of *dronc* promoter can emulate endogenous *dronc* transcriptional activity in the larval midgut and salivary gland.

Midguts and salivary glands were dissected from control and *dronc* promoter-reporter animals at the indicated times relative to puparium formation, homogenised and total RNA extracted for Northern blotting. Blots were probed with *LacZ*, *dronc* and *rp49* sequences. Endogenous *dronc* and 2.8 kb-*LacZ* transcript is not present in third instar larval midguts but are both upregulated following the late larval ecdysone pulse, suggesting that 2.8 kb of *dronc* promoter is sufficient to direct correct reporter expression in this tissue. Similarly, 2.8kb-*LacZ* expression increases following the late prepupal ecdysone pulse, as does endogenous *dronc*.

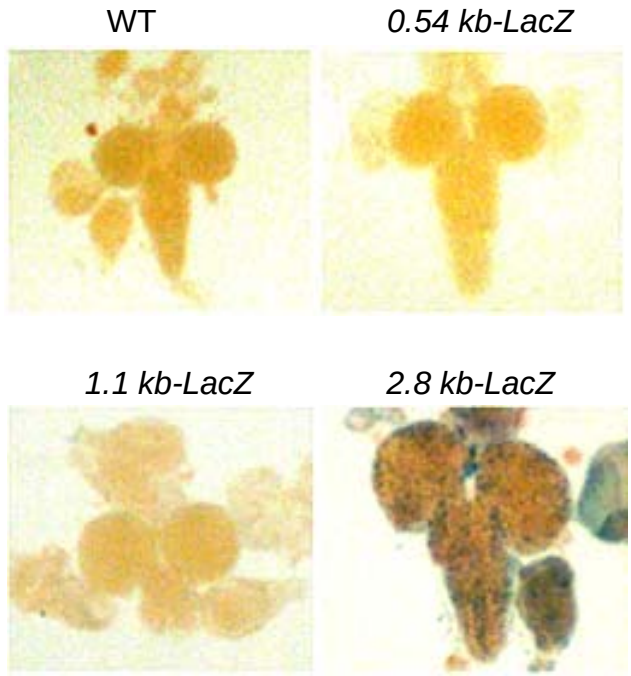


Figure 4.6 Expression of various *dronc* promoter-*LacZ* transgenes in larval brain lobes.

Brain tissue was dissected from late third instar larvae and stained with X-gal. No staining was observed in control, 0.54 kb- or 1.1 kb-*LacZ* brain tissue. Endogenous *dronc* is expressed in the larval brain and only the 2.8 kb-*LacZ* transgene has activity in this tissue, therefore 2.8kb of the *dronc* promoter contains the required regulatory elements to direct tissue-specific *dronc* expression in the brain.

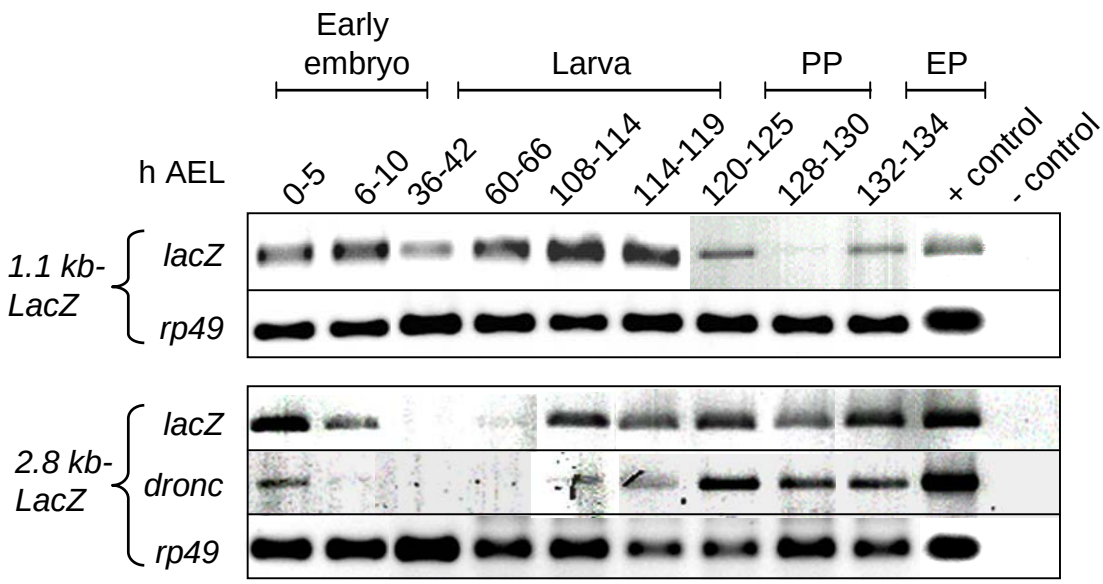
4.5 Temporal regulation of the *dronc* promoter

In an effort to ascertain the promoter requirement for correct temporal regulation throughout *Drosophila* development, expression of the *LacZ* transgene was compared with that of endogenous *dronc* in whole animals. An analysis of *dronc* and *LacZ* transcript during development suggested that the gross pattern of 2.8 kb promoter-driven *LacZ* expression is similar to that of endogenous *dronc* (figure 4.7). Consistent with the embryo X-gal staining, 1.1kb- and 2.8 kb-*LacZ* transcript is present in embryos. However, while 2.8 kb reporter activity drops off through the first and second larval instars, as does endogenous *dronc*, the 1.1 kb sustains expression through these developmental stages (figure 4.7). It was noted that 2.8 kb-*LacZ* is upregulated in response to the mid-third instar larval ecdysone pulse that triggers larval wandering behaviour, while endogenous *dronc* primarily is upregulated at the larval-puparium transition. This suggests that 2.8 kb of *dronc* promoter contains most elements required for the temporal regulation of *dronc* expression, however, while emulating *dronc* expression in midgut and salivary glands, 2.8 kb-*LacZ* may require additional elements for correct temporal expression in other tissues.

To further understand the temporal dynamics of 1.1 kb and 2.8 kb reporter construct expression, whole animal CPRG assays were performed on animals at various stages of development (figure 4.8). Consistent with low *dronc* expression, 2.8 kb promoter-*LacZ* driven βgal expression was low in second instar larvae, which increased significantly at 96 h AEL, followed by a sharp decrease and then steady increase from puparium formation, presumably in response to the late larval pulse of ecdysone which initiating metamorphosis. The 1.1 kb-*LacZ*-driven expression of βgal

Figure 4.7 Expression of endogenous *dronc* and the promoter-*LacZ* transgenes in animals at various stages of development.

RT-PCR analysis of *LacZ*, *dronc* and *rp49* expression is shown. Developmental stages are indicated as h AEL. Both the 1.1 kb and 2.8 kb promoter-*LacZ* constructs respond to the late third instar larval and the prepupal ecdysone pulses, which direct endogenous *dronc* expression. 22-25 cycles of PCR was carried out using cDNA prepared from animals at various stages of development and gene-specific primers. Note that the expression pattern of the 2.8 kb-*LacZ* transgene is similar to that of endogenous *dronc*. However, the expression of the 1.1 kb-*LacZ* transgene is deregulated and can be clearly seen in early larval stages when endogenous *dronc* expression is very low. In prepupal (PP) and early pupal (EP) stages, the 1.1 kb-*LacZ* transgene expression is lower, presumably due to a lack of expression in salivary glands. No expression for the 0.54 kb-*LacZ* transgene was observed at any stage. The positive (plasmid DNA) and negative (-cDNA) PCR controls are shown in the last two lanes of each panel.


Early embryo
Larva
PP
EP
h AEL
0-5
6-10
36-42
60-66
108-114
114-119
120-125
128-130
132-134
+ control
- control
1.1 kb-LacZ
lacZ
rp49
2.8 kb-LacZ
lacZ
dronc
rp49

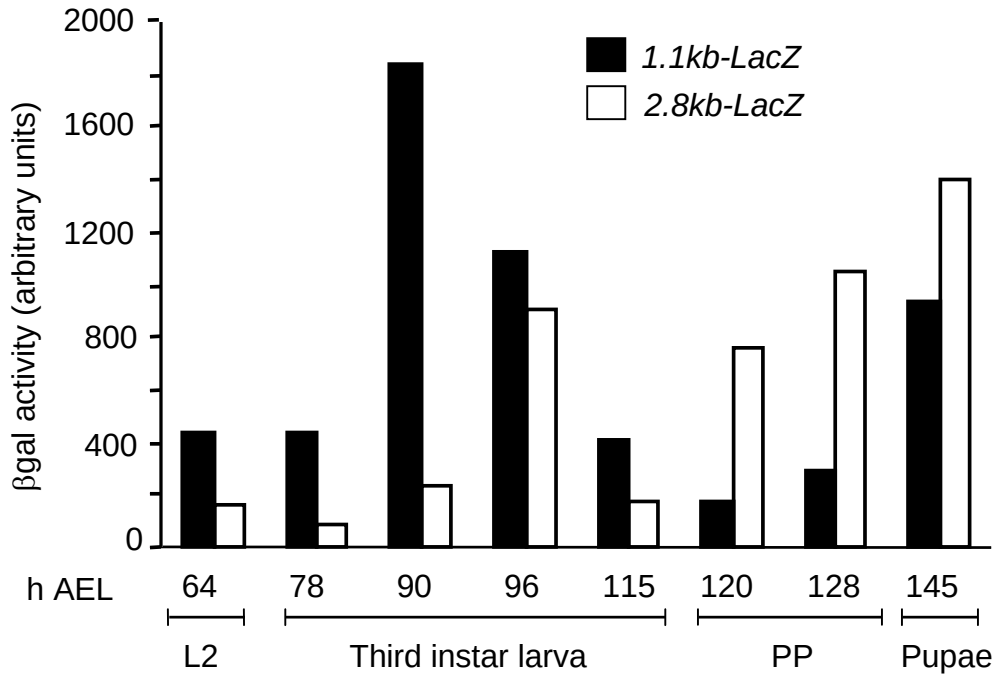


Figure 4.8 Quantitative assessment of the *dronc* promoter-*LacZ* transgene temporal expression profile.

βgal activity was determined in larvae, prepupae (PP) and pupae from 1.1 and 2.8 kb-*LacZ* transgenic animals. The 1.1 kb promoter driven *LacZ* expression is much higher in early larval stages but has reduced activity through metamorphosis. The 2.8 kb-*LacZ* reporter has a differential temporal profile compared to 1.1 kb, having low activity through the larval stages and increasing through metamorphosis. L2, second instar larvae; PP, prepupae.

was somewhat higher in second instar larvae, consistent with the RT-PCR and high midgut X-gal staining data, peaking at 90 h AEL, and then declining thereafter until the prepupal ecdysone pulse. The elevated activity of the 1.1 kb promoter at 90 h AEL suggests this region of the promoter lacks a control mechanism normally required to suppress *dronc* expression at this stage of development. Thus the data suggests the region of *dronc* promoter between 1.1 and 2.8 kb to be regulated by a temporally controlled transcriptional repressor, most notably functioning in the midguts. This is supported by the observation that the 2.8 kb promoter is less responsive to the mid third instar larval ecdysone pulse initiating larval wandering.

4.6 The Role of E74A, BR-C and E93 in the regulation of *dronc* expression

To analyse the roles of ecdysone-induced genes in regulating *dronc* expression, 2.8kb *dronc* promoter-*LacZ* transgene expression was analysed when in a genetic background null for *E74A*, *BR-C* and *E93*. These three EcR/Usp-regulated factors are involved in the regulation of PCD during metamorphosis, in particular the removal of the larval midguts and salivary glands. Midgut destruction commences at the beginning of puparium formation ~13 hours earlier than the removal of the salivary glands. Midguts from larvae that were transgenic for the 2.8 kb reporter construct and null mutant for one of the above transcription factors were dissected out from late third instar larvae (-12 h PRF) and early prepupae (+1 h RPF) and stained for βgal activity. From animals of similar genetic compositions, salivary glands were dissected from late third instar larvae (-12 h PRF), early prepupae (+1 h RPF), and early pupae (+12 h RPF). The requirement of these transcription factors in combination with the

2.8 kb promoter sequence to direct *LacZ* expression in these two tissues was assessed by βgal activity, which represents *dronc* expression.

Two *BR-C* mutant alleles, npr^1 (Belyaeva et al., 1980) and rbp^5 (Restifo and White, 1991) were used in combination with the 2.8 kb-*LacZ* transgene. npr^1 mutants are null for all BR-C isoforms and fail to initiate pupariation (Belyaeva et al., 1980). The rbp^5 mutant is null for the BR-C Z1 protein isoform, shows a defect in the removal of the larval salivary gland, and fails to develop beyond early pupae (Bayer et al., 1997; Kiss et al., 1988; Kucharova-Mahmood et al., 2002). When the 2.8 kb-*LacZ* transgene was present in the rbp^5 mutant background, there was no significant effect on its expression in the midgut at either the late third instar larval or early prepupal stages (figure 4.9), the staining pattern and intensity appearing similar to 2.8 kb-*LacZ* activity in the WT background. Similar results were obtained when the 2.8 kb-*LacZ* transgene was present in a *BR-C* null (npr^1) background in late third instar larval midguts (figure 4.9). Midguts from later stages of development could not be analysed because npr^1 mutants do not develop beyond the late third instar stage. Thus *dronc* expression, when under the control of 2.8 kb of promoter, is independent of BR-C in the midgut.

Unlike in the midgut, the expression of the 2.8 kb-*LacZ* transgene was practically absent in salivary glands from early pupal stages in the rbp^5 mutant (figure 4.10). Salivary glands from late larval stage npr^1 mutants had no detectable βgal staining (figure 4.10). These glands were incubated for extended periods in X-gal solution to detect low levels of staining. These data suggest that BR-C is essential for proper expression of *dronc* in salivary glands.

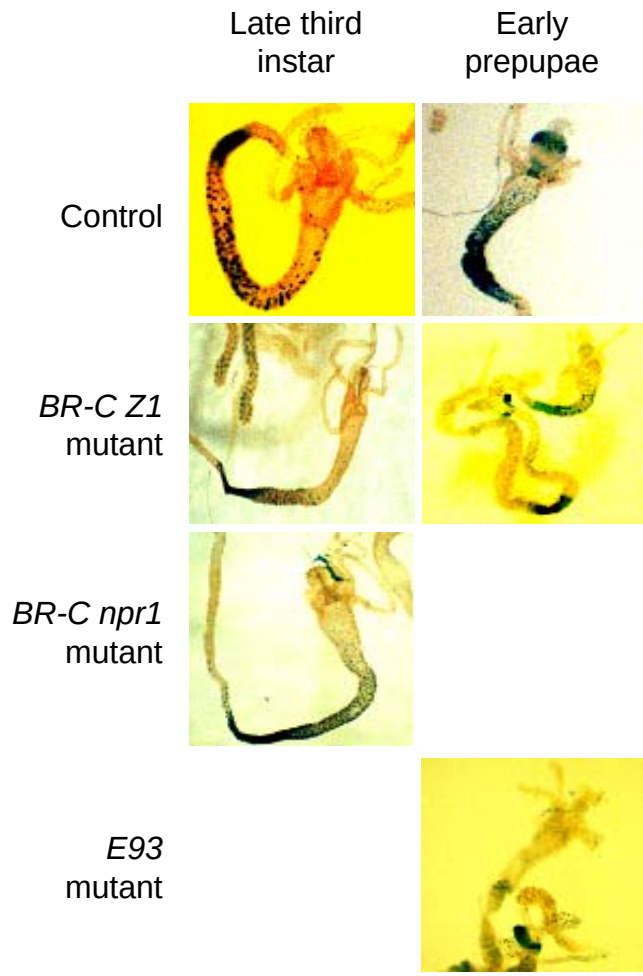


Figure 4.9 Expression of the 2.8kb *dronc*-promoter-*LacZ* transgene in midguts from *BR-C* and *E93* mutants.

The midguts were dissected out from late third instar larvae (-12 h RPF) and early prepupae (+1 h RPF) and stained with X-gal. In *E93* mutants, staining in midguts is reduced or absent (bottom panel) whereas *BR-C* mutation has limited impact on reporter expression.

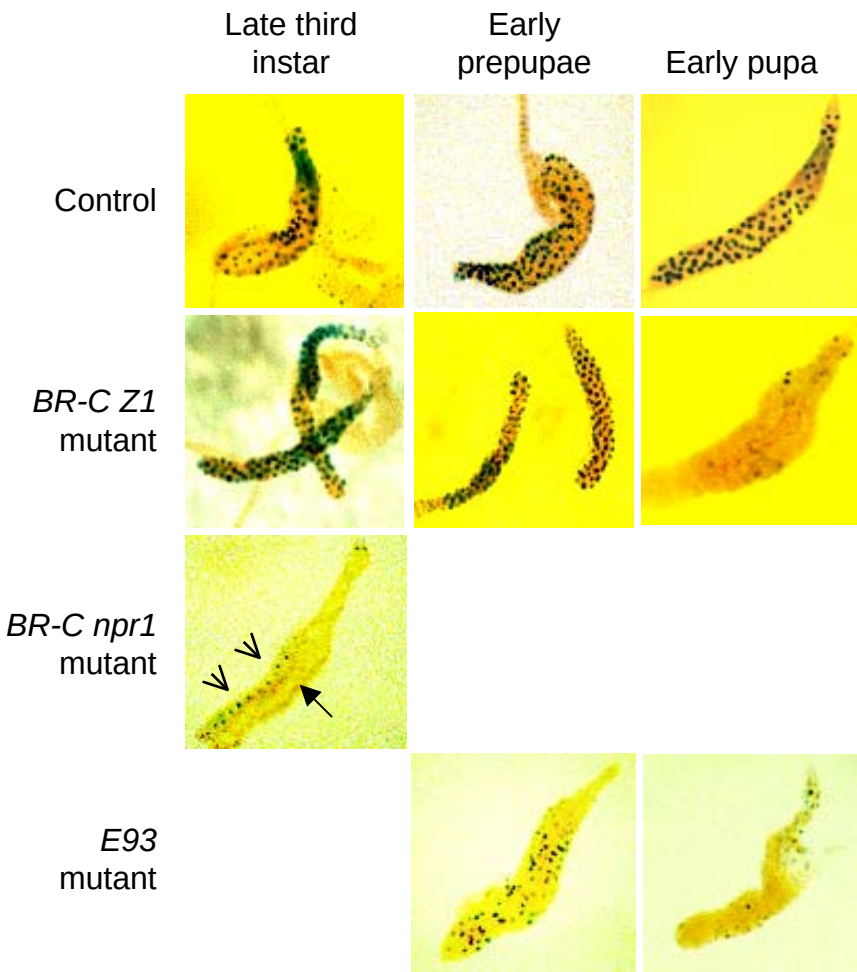


Figure 4.10 Expression of the 2.8kb *dronc*-promoter-*LacZ* transgene in salivary glands from *BR-C* and *E93* mutants.

Salivary glands were dissected out from early pupal stage (+12 h RPF) animals and stained with X-gal. βgal staining seen in *BR-C npr1* salivary gland panel (late third instar) represents fat body (arrow heads), while the salivary gland itself (arrow) is not stained. In *E93* mutants, staining in salivary glands is reduced or absent (bottom panel).

The *E93* gene encodes a novel nuclear protein and is specifically required for ecdysone induced cell death in both midgut and salivary glands (Lee et al., 2000). In *E93* null mutants, the larval midgut and salivary glands persist into early pupal stages when development is arrested (Lee et al., 2000). When the 2.8 kb-*LacZ* transgene was introduced into an *E93* mutant background, the expression of βgal was strongly suppressed in all regions of the midgut from early prepupal animals (figure 4.9). βgal activity in the salivary glands of early prepupal *E93* mutants was considerably lower and unevenly distributed compared to controls while only a few cells with βgal staining were present in the salivary glands of early pupal (+12 h RPF) *E93* mutant animals (figure 4.10). These data demonstrate that E93 is required for the maximal expression of *dronc* during PCD in the midgut and salivary glands.

The effect of *E74A* mutants on the expression of the 2.8 kb-*LacZ* transgene was also analysed. Consistent with previous findings (Lee et al., 2002b), significant qualitative or quantitative changes in X-gal staining in these salivary glands were not observed.

To compare the expression of the 2.8 kb-*LacZ* reporter transgene to that of endogenous *dronc* in the salivary glands of the *BR-C*, *E93* or *E74A* transcription factor mutant salivary glands, animals were staged to the time preceding salivary gland destruction (+12 h RPF) and salivary glands were dissected for RNA extraction. RT-PCR on cDNA from these glands showed 2.8 kb-*LacZ* transgene activity in *E74A* null animals comparable to controls (figure 4.10 C). This data suggests that E74A does not play a crucial role in regulating *dronc* expression through 2.8 kb of *dronc* promoter during salivary gland cell death.

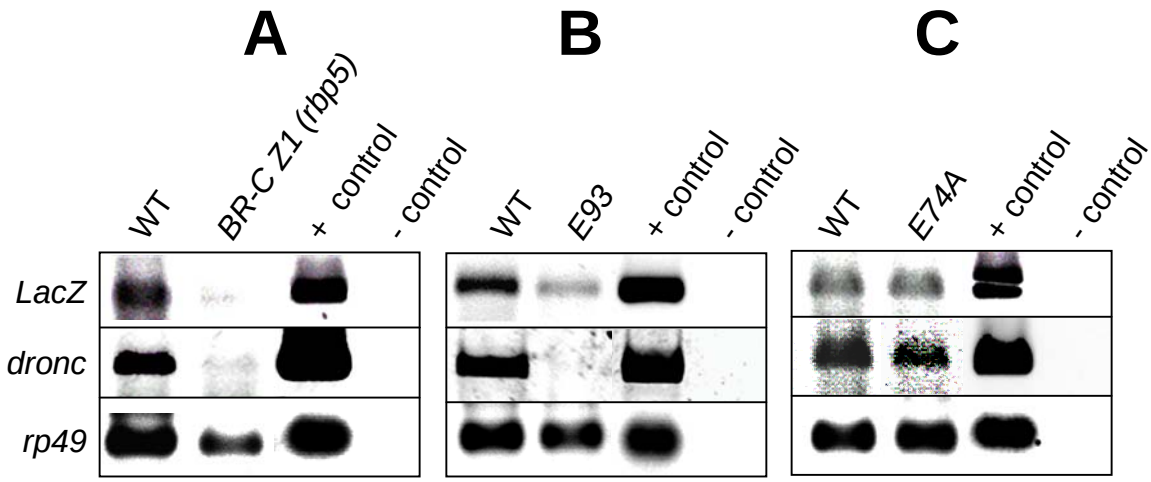


Figure 4.11 RT-PCR analysis of *LacZ* and *dronc* expression in the salivary glands of 2.8 kb promoter-*LacZ* transgenic flies in *BR-C Z1* (*A*), *E93* (*B*) and *E74A* (*C*) null backgrounds.

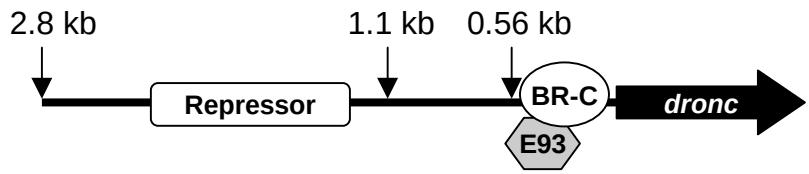
Salivary glands were dissected out from animals +12 h RPF. Reporter activity was reduced in *E93* and *BR-C* mutant salivary glands. *Rp49* expression was used as a control. 2.8 kb promoter-*LacZ* transgene was in either the WT or mutant background as indicated. The positive (plasmid DNA) and negative (-cDNA) PCR controls are shown in the last two lanes of each panel.

dronc and 2.8 kb-*LacZ* transcript was barely detectable in salivary glands from 13 h RPF *rbp*^{5} animals consistent with the above βgal staining patterns and previous data regarding 2.8 kb-*LacZ* and *dronc* expression respectively (figure 4.10 A). *dronc* transcript is absent in the *E93* mutant salivary glands at a time when it is otherwise expressed in the WT salivary glands, whereas 2.8 kb-*LacZ* transcript is present but at very low levels (figure 4.10 B). The low 2.8 kb reporter transcript level is reflected in the low βgal activity in the *E93* mutant salivary glands (figure 4.10) demonstrating a dependence of the 2.8 kb reporter activity in the salivary glands on the level of the E93 transcription factor.

4.7 Discussion

It is evident from the data presented in chapters 3 and 4 that the expression of *dronc* transcript during development is highly dynamic. As summarised in figure 4.11, the results reported in this chapter (now published) provided the first analysis of the *dronc* promoter and form a basis for further studies, some of which are outlined in chapter 5. *dronc* is regulated both temporally and spatially by the steroid hormone ecdysone and thus the *dronc* promoter can be exploited when studying components of the regulatory apparatus mediating gene expression by nuclear hormones.

Experiments in this chapter demonstrate that 2.8 kb of the *dronc* promoter is sufficient to emulate the temporal expression profile of endogenous *dronc* throughout development. Previous experiments have shown that *dronc* is predominantly expressed in the larval midgut and salivary glands, and the central nervous system (Dorstyn et al., 1999a). 2.8 kb of the *dronc* promoter contains most of the promoter elements for the correct spatial regulation of *dronc* expression in these tissues. This region harbours a midgut-specific repressor element important to keep *dronc* levels low during periods when ecdysone titres are low such as the late third instar larval period preceding puparium formation. The promoter requirements for transcription are markedly different in the midgut with the 1.1- 2.8 kb region not being required for transcription in this tissue. These results clearly demonstrate that distinct regions of the *dronc* promoter are required for expression in specific tissues. Given the relationship between core cell death gene expression and key transcription factor expression, the promoter analysis reported in this chapter suggests that different transcription factors regulate *dronc* expression in a tissue-dependent manner.



	SG	MG	BL	OV
2.8 kb-LacZ	+	+	+	+
1.1 kb-LacZ	-	+	-	+
0.56 kb-LacZ	-	-	-	-

Figure 4.12 A summary of the *dronc* promoter analysis.

Potential BR-C and E93 binding elements and a repressor-binding site are indicated. Lower panel summarises the results of *dronc* promoter-*LacZ* expression in various tissues. SG, larval, prepupal and early pupal salivary glands; MG, larval or prepupal midgut; BL, larval brain lobes; OV, adult ovaries.

Preliminary data demonstrated there to be an EcR/Usp binding site between 1.1 and 2.3 kb of the *dronc* promoter, and *in vitro* experiments showed this element was important in regulating *dronc* expression. The role of the Ecdysone Receptor in *dronc* expression is the subject of chapter 5 of this thesis.

This data support previous work demonstrating a direct link between a hormone signal and a tissue-specific programmed death response (Cakouros et al., 2002; Jiang et al., 2000). Using a transgenic *LacZ* promoter-reporter strategy, Jiang and colleagues (2000) showed 1.3kb of *rpr* promoter was required for correct temporal salivary gland expression. The BR-C Z1 isoform, essential for salivary gland destruction and maximal *dronc* expression in the salivary glands, was also required for salivary gland specific expression of the *rpr* and *hid* death activators, however, *E74A* mutants had only marginal effects on *hid* expression (Jiang et al., 2000; Lee et al., 2002b; Zhimulev et al., 1995). Experiments conducted by Cakouros et al. (2002) demonstrated that ecdysone induced *dronc* expression, caspase activation, and cell death in *l(2)mbn* cells, all prevented by *dronc*-specific RNAi treatment. It was also shown using EMSA analysis followed by cold oligo competition experiments that BR-C isoforms specifically bound the *dronc* proximal promoter following ecdysone treatment. The BR-C Z1 isoform could transactivate the *dronc* proximal promoter, a response abrogated by BR-C RNAi treatment, which also reduced *dronc* expression following ecdysone treatment. These data show that normal ecdysone-induced *dronc* expression and apoptosis is BR-C-dependent. Furthermore, EMSA experiments using nuclear extracts from *BR-C Z1* mutant animals (rbp^5) show loss of ecdysone-inducible complexes on the *dronc* proximal promoter. The work in this chapter complements and extends the work by

Cakouros and colleagues (2002) in regard to the requirement of BR-C Z1 for the stage- and tissue-specific expression of *dronc* throughout *Drosophila* development in multiple tissues.

Minimal promoter requirements for *dronc* reporter expression in midguts is markedly different to that in the salivary glands, supporting previous data showing tissue-specific transcription factor requirements for core cell death genes between the midgut and salivary glands (Lee et al., 2002a; Lee et al., 2002b; Lee et al., 2000). For example, *BR-C* mutation does not impact the transcription of endogenous *dronc* or 2.8 kb-*LacZ* in the midgut nor does it reduce midgut TUNEL staining. However, unlike $dronc^{d5}$ mutants, midgut destruction in BR-C mutants is arrested at the early prepupal stage and no typical autophagic structures are observed (Lee et al., 2002a).

In the salivary glands, *E93* mutants maintain plasma membrane integrity and cytoplasmic vacuoles similar to that observed in $dronc^{d5}$ salivary glands, whereas *BR-C* mutants lack these structures, however both of these transcription factor mutations disrupt *dronc* expression. *E93* mutation selectively disrupts *dronc* expression in the midguts, and a number of cell death genes in the salivary glands, an observation supported by the reduced 2.8 kb-*LacZ* expression in these tissues in *E93* mutants (figures 4.9 and 4.10) (Lee et al., 2002a; Lee et al., 2002b; Lee et al., 2000). *E93* mutant midguts fail to reach advanced stages of histolysis, have incomplete autophagy, but have TUNEL-positive nuclei (Lee et al., 2002a). The fact that $dronc^{d5}$ mutants have more advanced midgut destruction but TUNEL-positive nuclei and variable aspects of autophagy, suggests the transcriptional dependence on *E93*

expression and of other unknown factors apart from *dronc* are needed for complete midgut destruction.

Regulation of midgut destruction is complex and multifactorial in regard to specific functions of the death pathway components. However, the fact that *dronc* and the 2.8 kb-*LacZ* transgene are markedly upregulated in the late larval midgut suggests a critical role for this caspase in midgut removal. The data presented in chapter 3 suggest, not only that DRONC is not required for many aspects of midgut histolysis, but that effector caspase activation also can occur independently of DRONC in this tissue. Thus, while the mechanism behind the developmental transcriptional regulation of *dronc* has been further explored, why there is significant functional redundancy in the midgut remains unclear.

This chapter established the minimal *dronc* promoter required for spatial and temporal *dronc* expression is within the first 2.8 kb. This region is important for both BR-C- and E93-mediated *dronc* transcription in salivary glands and E93-mediated *dronc* transcription in the midgut. Furthermore, the 1.1-2.8 kb promoter region harbours elements important for salivary gland expression along with a putative midgut-specific repressor element. Only the 0.54-1.1 kb promoter region is required for expression in the midgut. The identification of the regulatory functions of these promoter regions formed the basis of experiments designed to identify factors necessary for the tissue- and stage-specific regulation of *dronc* expression during PCD.

Chapter 5

Identification of regulatory elements controlling the temporal and spatial expression of dronc

5.1 Introduction

The major components of the core cell death machinery are largely known. The general relationships between key upstream transcription factors and the host of cell death activators and caspases has also been demonstrated. However, the precise mechanism by which the key upstream regulators communicate with and transduce the death signal to the core cell death executors remains to be fully defined.

dronc was the first caspase shown to be transcriptionally regulated by the steroid hormone ecdysone (Dorstyn et al., 1999a). Regulation of DRONC activation requires the adapter molecule DARK in concert with the blocking of IAP function by the H99 death activators (Chen et al., 1996; Grether et al., 1995; White et al., 1994; Wilson et al., 2002). Thus, multiple pre- and post-transcriptional and direct and indirect control points are involved in regulating the correct temporal and spatial timing of *dronc* expression and activation. Chapters 3 and 4 demonstrated hormone-induced transcriptional regulation to be complex and involve multiple transcription factors working in a coordinated manner with the EcR/Usp nuclear receptor heterodimer.

There are three EcR isoforms in *Drosophila*, EcR-A, B1 and B2 (Yao et al., 1992). These isoforms are highly homologous in the DNA and ligand binding domains but differ in their amino terminal transactivation domain. The EcR-B1 isoform is predominantly expressed in tissues destined to undergo PCD whereas the EcR-A isoform is expressed in tissues that differentiate in response to ecdysone (Talbot et al., 1993; Yao et al., 1992). EcR/Usp and the ecdysone-induced transcription factors

βFTZ-F1, BR-C, E74, E75 and E93 play critical roles in the ecdysone-destruction of the larval salivary glands and midgut. For example, EcR/Usp directly regulates *rpr* transcription in salivary glands and BR-C is required for maximal *rpr* and *dronc* expression (Cakouros et al., 2002; Jiang et al., 2000). BR-C and E74A are also required for the optimal induction of *hid* in salivary glands (Jiang et al., 2000). In *E93* mutant salivary glands, *rpr*, *hid*, *ark* and *dronc* mRNA levels are severely reduced (Lee et al., 2000). This indicates that ecdysone-mediated regulation of death molecules such as *rpr*, *hid*, *dark* and *dronc* is crucial for correct PCD in salivary glands and midgut during *Drosophila* metamorphosis. The importance of direct transcriptional regulation of caspase levels therefore appears to be a fundamental element in the initiation and control of PCD *in vivo* rather than the activation of constitutively present death machinery.

This chapter aims to clarify the role of the EcR/Usp in regulating *dronc* temporal/spatial expression at the level of the *dronc* promoter sequence. The preliminary promoter deletion studies in Chapter 4 showed that *dronc* transcriptional regulation is complex and involves both temporal and spatial control (Daish et al., 2003). This chapter presents work demonstrating that EcR-B1/Usp directly binds and transactivates the *dronc* promoter and that this binding is necessary for correct tissue specific *dronc* expression. These studies provide a basis for investigating spatially/temporally regulated gene transcription of a caspase during development by a steroid hormone receptor.

Results

5.2 Analysis between 2.8 and 1.1kb of *dronc* promoter

As demonstrated in the previous chapter, the region of *dronc* promoter between 1.1 kb and 2.8 kb contains elements important for the tissue-specific expression of the dronc promoter-reporter *LacZ* gene. Consequently this region was further dissected by promoter-reporter transgene generation. Three promoter deletions were generated dividing the promoter region between 1.1 kb and 2.8 kb into four segments (figure 5.1 A and B). Transgenic flies homozygous for these constructs were generated for βgal activity analysis in the larval midgut and salivary glands using X-gal staining and βgal quantitative assays.

5.3 A region of the *dronc* promoter contains a putative midgut repressor

X-gal staining of the midguts from animals containing the promoter-reporter constructs dissecting the region between 1.1 kb and 2.8 kb showed that promoter regions between 2.3 kb and 2.8 kb had regulatory elements important for gastric caecae-specific expression (figure 5.2 A). Only the 2.8 kb reporter lacked significant gastric caecae staining as well as reduced staining intensity throughout the midgut body. Quantitative analyses demonstrated that all promoter constructs with more than 1.1 kb promoter showed increased activity in response to the late pupal ecdysone pulse with all constructs having greater activity levels than the 2.8 kb reporter, before or after the larval-pupal transition (figure 5.2 B), consistent with the X-gal staining. This indicates that basic ecdysone response elements are present in these promoter

Figure 5.1 An ecdysone receptor binding consensus sequence in the *dronc* promoter.

(A) *dronc* promoter sequence between 1.1kb and 1.64 kb. An EcRBE consensus sequence is positioned just distal to the primer position used to generate a 1.33 kb-*LacZ* promoter-reporter transgene. Underlined sequence indicates position of primers used to generate promoter-reporter lengths indicated to the left of the sequence. The candidate EcRBE consensus sequence is boxed. **(B)** *dronc* promoter-reporter constructs used to make transgenic flies to assess the regulatory function of the sequence between 1.1 kb and 2.8 kb. Primers indicated in (A) were used in combination with a common reverse primer to generate *LacZ* promoter-reporter transgenes using the pCasper-NLS-LacZ p-element transformation vector (a gift from C. Thummel).

A

CAAAAGGGTATTATATTTGTATTCGAATTTGTATGTAATTCTGAT

1.64kb CGTATGTACGTTATGTTATCGTAAGTGTACCATCCTAGATCCTCT

AATGAATTGAATTGAAATGAAGCCAACTCTGTCTCTTGTGATTC

TATTGTGATGCTATGGGAGTCAAGCAGCACTCAACTGTTTTTAT

GCCTCTTAAAGTTTAAAACAAAGCTAATCGTAGCAAAACCTAAA

ACTAAACTACCTATCTCTACACTAGTCTATGCAATGCTAATTGG

CAGCCCCAACAATTGAAAACAATCATGCACAATAAATGTTATGC

EcRBE AATTGAGAGAATCCAAAGGCCAAAGACCCACAACTTTTTCTTAC

1.33kb ACTACTCGAAGTAATTGTGTACAACTAAAGGAATTCTAGATTTT

AAATGTGCTAAAACTGTTTATAGTCAAGTACTTAATTAAGAGTG

TAATTATCAAATATTAATACGTACAAAGGTTGGGGCATTTGGCA

TAGTAATACTAATAATGTATAAACGTGGGATCCGATGATTTGAT

TGATGATAATGGAGCGGTGCAAATGCGTGTGACAATGTACGTA

TGTAACAAACAATTGAATTTAAATGTTTAATAAATGTGTGGAAA

1.1kb TGAATAAATGCTCTGTGTGCTAAATTCCTCAATTACTCAATAA

B

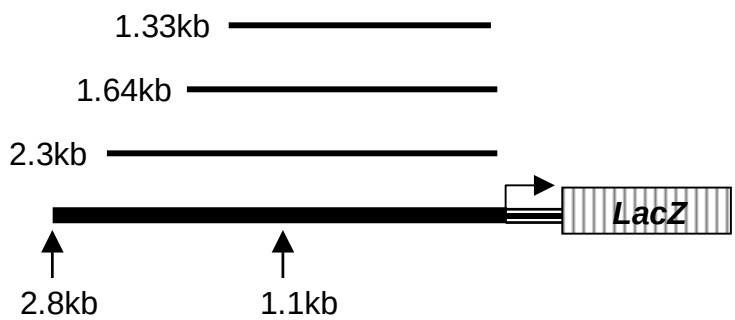
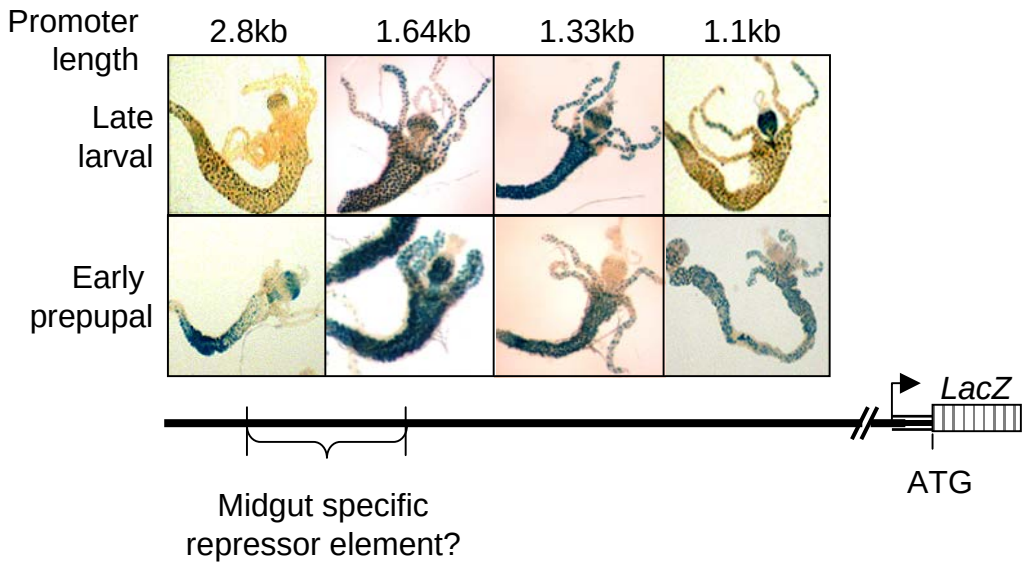


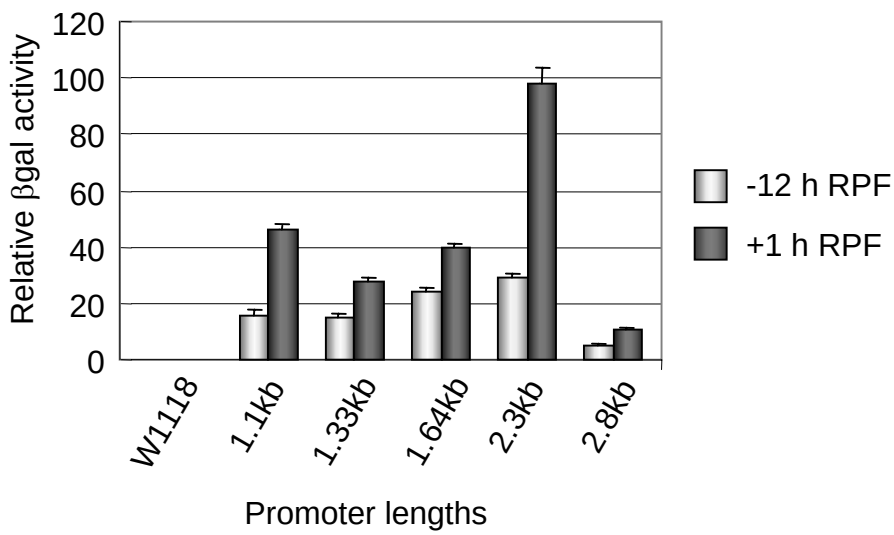
Figure 5.2 A region of the *dronc* promoter is required for spatial and quantitative regulation of expression in the midguts.

(A) βgal staining of midguts from *dronc* promoter-reporter transgenic *Drosophila* is shown. Tissues were dissected out at indicated developmental stages and stained with X-gal. The figure shows qualitatively similar staining patterns for 1.64 kb and 1.33 kb transgenes with expression throughout midgut and gastric caecae. **(B)** CPRG assay of midguts from *dronc* promoter-reporter deletion transgenic animals. Sequence between 2.3-2.8 kb appears to harbour elements required for repressing reporter transcription.

A


Promoter length
2.8kb
1.64kb
1.33kb
1.1kb
Late larval
Early prepupal
LacZ
ATG
Midgut specific repressor element?

B


Relative βgal activity
120
100
80
60
40
20
0
W1118
1.1kb
1.33kb
1.64kb
2.3kb
2.8kb
Promoter lengths
-12 h RPF
+1 h RPF

regions to drive reporter transcription in the midguts and that a midgut-specific repressor element(s) reside between 2.3 kb and 2.8 kb. The most notable post-ecdysone pulse increase is shown by the 2.3 kb reporter suggesting this has unique elements to maximise the transcriptional response when repression is relieved by deletion of the sequence between 2.3-2.8 kb.

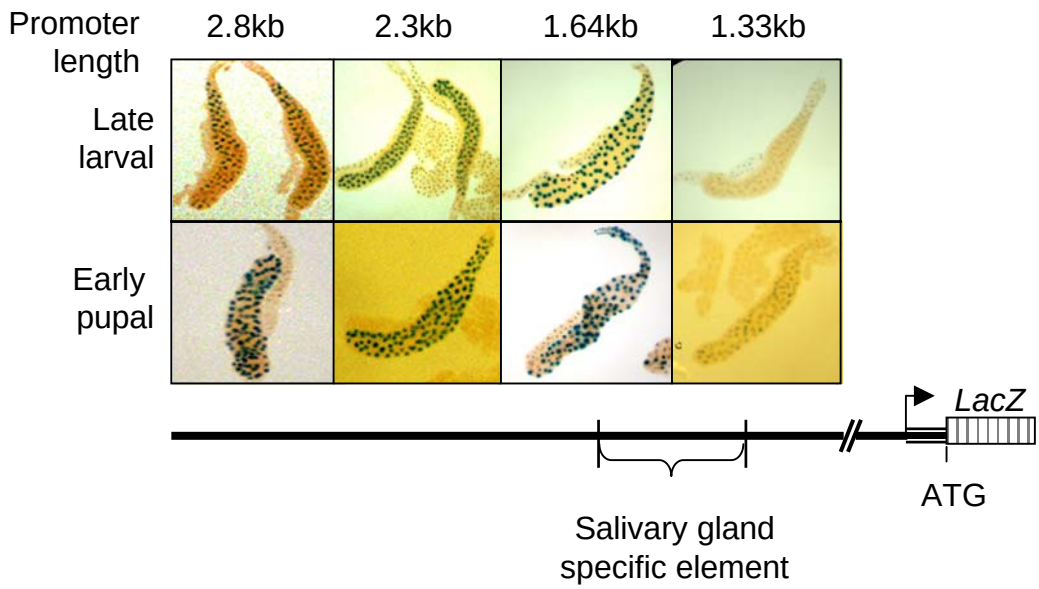
5.4 A region of the *dronc* promoter is required for the spatial regulation of *dronc*

Salivary glands were dissected from wandering third instar (-12 h RPF) and early pupal (+12 h RPF) animals and stained with X-gal to observe reporter activity. Staining revealed a salivary gland specific element located between 1.33 kb and 1.64 kb of *dronc* promoter (figure 5.3 A). Low level staining was possible to visualise in the salivary glands from 1.33 kb-*LacZ* +12 h RPF animals, suggesting a basal response to the late prepupal ecdysone pulse, however, expression of the 1.64 kb-, 2.3 kb- and 2.8 kb-*LacZ* transgenes was significantly greater (figure 5.3 A, lower panel). Quantitative analysis revealed that only the 2.3 kb and 2.8 kb reporter transgenes had significant transcriptional responses to the late prepupal ecdysone pulse (figure 5.3 B) compared to the 1.64 kb promoter transgene, which has deregulated high activity levels. This result indicates that salivary gland specific elements reside between 1.33 kb and 1.64 kb *dronc* promoter to regulate expression in this tissue while additional upstream elements are required to modulate the transcriptional response.

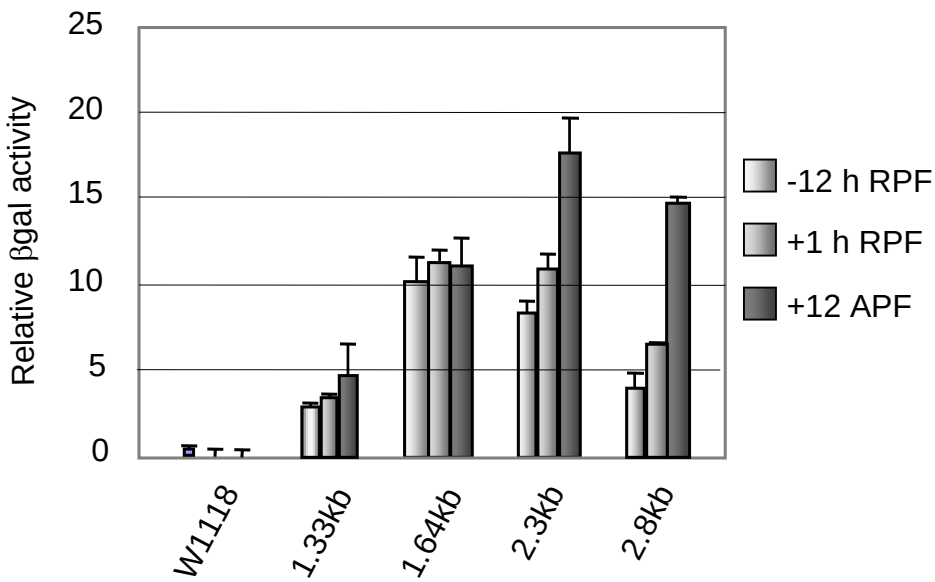
Figure 5.3 A region of the *dronc* promoter is required for spatial regulation of expression.

(A) βgal staining of salivary glands from *dronc* promoter-reporter transgenic *Drosophila* is shown. Tissues were dissected out at indicated developmental stages and stained with X-gal. The figure shows tissue specific expression between 1.64 kb and 1.33 kb transgenes in salivary glands demonstrating its requirement for salivary gland specific expression. **(B)** CPRG assay of salivary glands from *dronc* promoter-reporter deletion transgenic animals. 2.8 kb of promoter has an activity profile similar to endogenous *dronc* with the most marked relative increase in activity occurring at the prepupal/pupal developmental transition.

A


Promoter length
2.8kb
2.3kb
1.64kb
1.33kb
Late larval
Early pupal
LacZ
ATG
Salivary gland specific element

B


Relative βgal activity
25
20
15
10
5
0
W1118
1.33kb
1.64kb
2.3kb
2.8kb
-12 h RPF
+1 h RPF
+12 APF

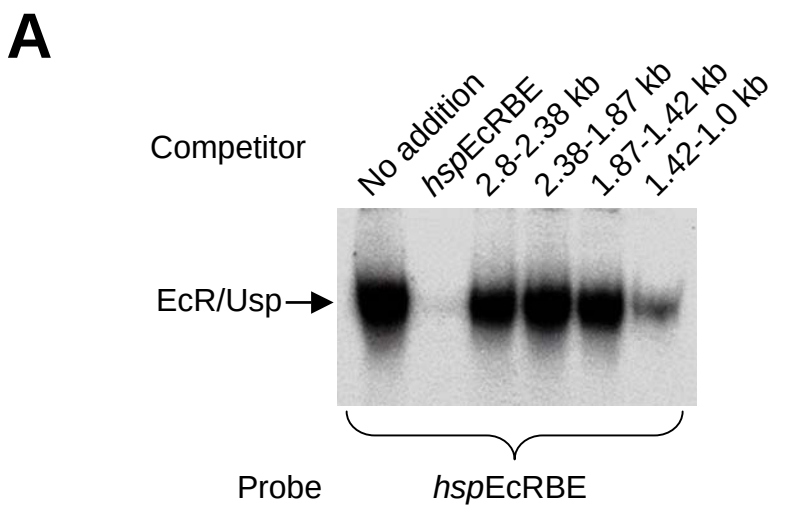
5.5 *dronc* promoter contains an EcR/Usp binding element (EcRBE)

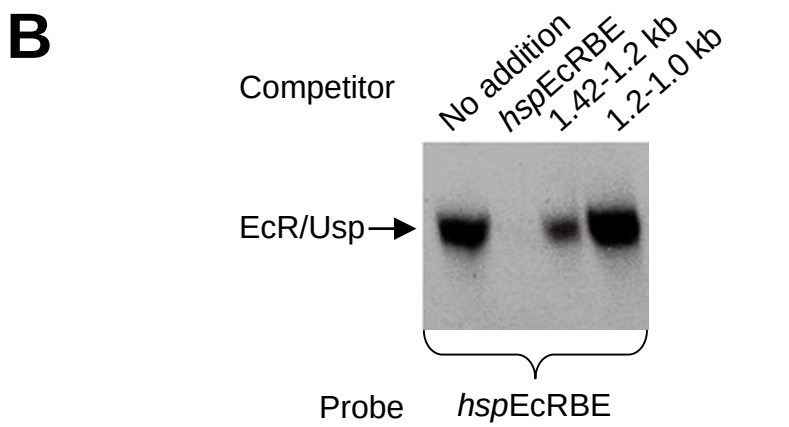
Jiang and colleagues (2000) demonstrated a direct binding relationship between EcR/Usp and the promoter of the death activator *reaper*. As the *dronc* promoter is directly regulated by BR-C and possibly E93 (D.Cakouros personal communication), and these transcription factors are directly regulated by EcR/Usp, it is possible that *dronc* promoter is also directly bound by the EcR/Usp complex and contributes to its timing or location of expression (Cakouros et al., 2002). EcR-B1 isoform is the predominant form in the larval salivary glands prior to their destruction therefore it is predicted to be involved in *dronc* regulation in this tissue.

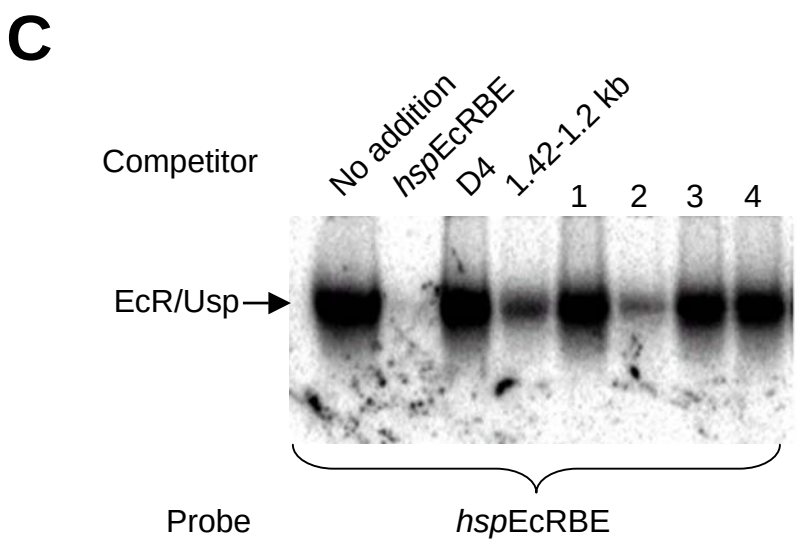
To screen for possible binding of EcR/Usp to the *dronc* promoter, electrophoretic mobility shift analysis (EMSA) and competition experiments were performed. EMSA experiments were carried out using the *hsp70* EcRBE (*hsp*EcRBE) as a probe and *in vitro* translated EcR/Usp protein (figure 5.4 A). The EcR/Usp complex was completely abolished when competed with the cold *hsp*EcRBE oligonucleotide demonstrating the specificity of the complex. 400 bp PCR products spanning the upstream *dronc* promoter region were used as competitors in an attempt to locate an EcR/Usp binding element. Competitors spanning the region between 2.8 kb to 1.42 kb had no significant effect on the EcR/Usp complex formation, however, the region between 1.0-1.42 kb clearly competed out most of the EcR/Usp complex (figure 5.4 A). Further experiments mapped the potential EcRBE to the region between 1.2-1.42 kb (figure 5.4 B). This ~200 bp region was analysed in more detail using overlapping 60 bp oligonucleotides. The second oligonucleotide spanning this sequence successfully competed out the EcR/Usp complex in EMSA,

Figure 5.4 *dronc* promoter harbours a putative EcR/Usp binding element (EcRBE).

(A) In vitro translated EcR and Usp proteins were incubated with the EcR consensus probe hspEcRBE for 20 min in the presence of *dronc* promoter fragments. 400 bp PCR fragments spanning the *dronc* promoter region from 2.8 kb to 1.1 kb were gel purified and 400 ng used in each reaction. A positive control (*hsp*EcRBE) was used at 40 ng (equimolar). Complexes were resolved on an acrylamide/TBE gel, dried on 3MM Whatmann paper and exposed to Kodak film overnight. The EcR/Usp complex is indicated. **(B)** EMSA experiment was performed as in (A) except PCR fragments used as competitors spanned the regions from 1.42 kb to 1.0 kb. *hsp*EcRBE was used as the probe. **(C)** EMSA was performed as in (A). Negative control competitor (D4) corresponds to the *dronc* promoter region between 67 bp to 7 bp upstream of the transcription start site. Oligonucleotide competitors correspond to the *dronc* promoter region 1.44 kb to 1.2 kb upstream of the transcription start site. *Hsp*EcRBE was used as the probe.


A
Competitor
No addition
hspEcRBE
2.8-2.38 kb
2.38-1.87 kb
1.87-1.42 kb
1.42-1.0 kb
EcR/Usp
Probe
hspEcRBE


B
Competitor
No addition
hspEcRBE
1.42-1.2 kb
1.2-1.0 kb
EcR/Usp
Probe
hspEcRBE


C
Competitor
No addition
hspEcRBE
D4
1.42-1.2 kb
1
2
3
4
EcR/Usp
Probe
hspEcRBE

indicating that the potential EcRBE in the *dronc* promoter resides within this sequence (figure 5.4 C).

5.6 An EcR/Usp binding site resides in the *dronc* promoter

Sequence analysis of the 60 bp region identified above revealed a potential EcR/Usp binding site that had a 10/13 bp match with an EcRBE consensus sequence (figure 5.5 A). An oligonucleotide containing this potential EcRBE was used as a competitor along with a mutant oligonucleotide that had specific mutations in this binding site (figure 5.5 A). The EcR/Usp protein complexing with the labelled *hsp*EcRBE oligonucleotide sequence could be competed out with the cold *hsp*EcRBE oligonucleotide (figure 5.5 B). Increasing amounts of the *dronc*EcRBE oligonucleotide also competed out the EcR/Usp complex, however, higher amounts were needed to abolish binding of EcR/Usp to *hsp*EcRBE. The oligonucleotide containing the mutated *dronc*EcRBE consensus completely failed to compete out the *hsp*EcRBE-EcR/Usp complex (figure 5.5 B).

5.7 The EcR-B1 isoform specifically binds the *dronc* promoter

The EcR-B1 isoform is predominantly expressed in tissues destined to undergo PCD whereas the EcR-A isoform is expressed in tissues fated for morphogenetic changes to form adult structures (Talbot et al., 1993). It can therefore be predicted that *dronc*, which is expressed in tissues undergoing PCD, is likely to be regulated by the EcR-B1 isoform. In *l(2)mbn* cells, the EcR-B1 and EcR-B2 isoforms are highly abundant whereas EcR-A is not detectable. When EMSA analysis was

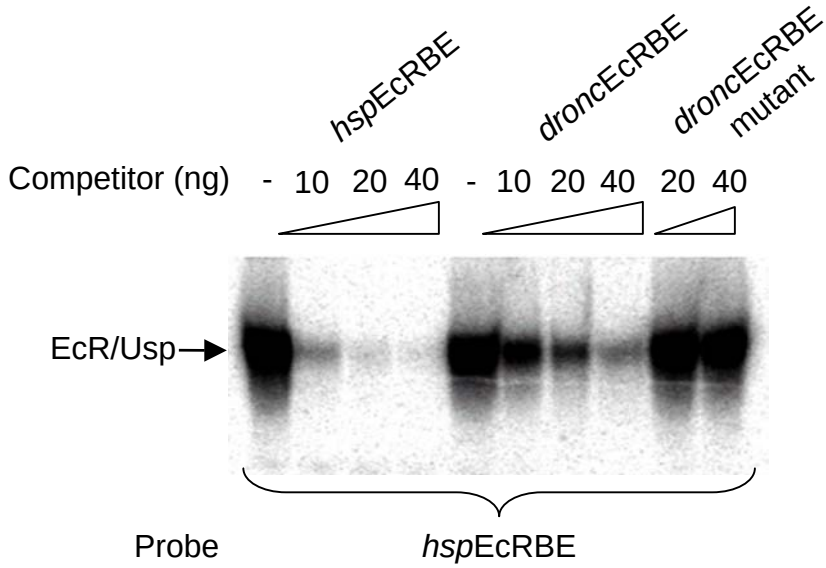
Figure 5.5 *dronc* EcRBE binds EcR/Usp.

(A) The EcR consensus binding site, its variations and the putative *dronc*EcRBE sequences are shown. The conserved residues in the *dronc*EcRBE are underlined. The asterisk (*) represents the residues that have been mutated in a mutant *dronc*EcRBE, which was used in the following studies. These sequences correspond to the oligonucleotides used in EMSA. **(B)** In vitro translated EcR and Usp proteins were incubated with the EcR consensus probe (*hsp*EcRBE) for 20 min in the presence of increasing amounts of *hsp*EcRBE competitor, *dronc* EcRBE competitor or *dronc* EcRBE mutant competitor oligonucleotides. Complexes were resolved on an acrylamide/TBE gel, dried on 3MM Whatmann paper and exposed to Kodak film overnight. The EcR/Usp complex is indicated.

A

EcRBE consensus	AGTTCAATGGCNT
	GTG GT CCG G
	C G AA
dronc EcRBE	AGGCCAAAGACCT
	* * *
dronc EcRBE mut	AAACTAAAGACCT

B

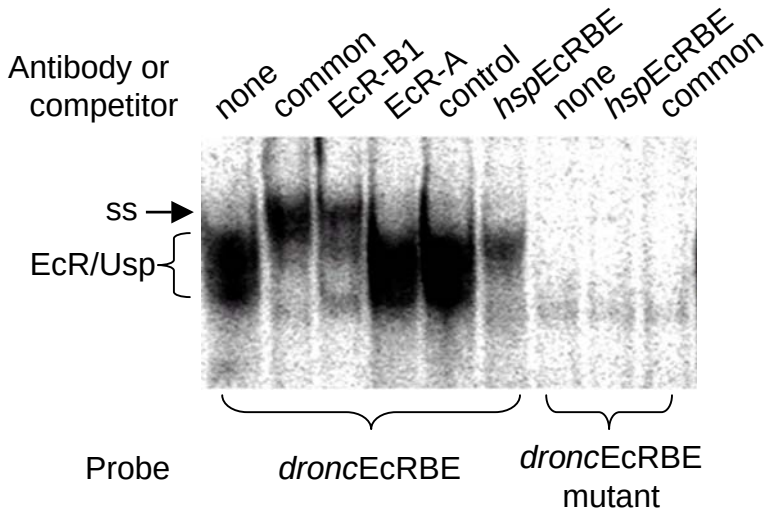


performed on *l(2)mbn* nuclear extracts using common and isoform specific EcR antibodies, EcR-A antibody failed to supershift a *dronc*EcRBE-EcR/Usp complex. When either the common or EcR-B1 specific antibody was added, this complex was supershifted indicating that the EcR-B1 isoform was binding to this consensus on the *dronc* promoter (figure 5.6 A). A cold *dronc*EcRBE oligonucleotide effectively competed with the formation of this complex. No EcR/Usp binding complex was observed to form when a probe containing a mutated *dronc*EcRBE sequence was incubated with the nuclear extracts (figure 5.6 A). These results clearly demonstrate that EcR-B1 isoform specifically is recruited to the *dronc* promoter. Because the EcR-B1 antibody effectively supershifted all of the *dronc*EcRBE-EcR/Usp complex, it can be presumed that EcR-B2 was not also contributing to its formation. RT-PCR analysis of EcR-B1 expression in *Drosophila* tissues shows it is expressed in salivary glands and midguts at developmental times preceding *dronc* expression (D. Cakouros personal communication). EMSA analysis on pre and early pupal nuclear extracts using the *dronc*EcRBE probe demonstrated *in vivo* formation of the *dronc*EcRBE-EcR/Usp complex at developmental stages when PCD is occurring (figure 5.6 B). This complex was not formed when the same extracts were incubated with the mutated *dronc*EcRBE probe (figure 5.6 B). Preliminary experiments using the EcR-B1 isoform specific antibody showed formation of a supershifted complex when added to whole prepupal lysates with the *dronc*EcRBE probe (figure 5.7 A). When this experiment was repeated with nuclear extracts from prepupal midguts and salivary glands, an EcR-B1 isoform specific supershifted complex was also formed (figure 5.7

Figure 5.6 EcR-B1 isoform specifically binds to the *dronc* promoter.

(A) Nuclear extracts prepared from *l(2)mbn* cells treated with ecdysone for 6h were incubated with the *dronc* EcRBE or the EcRBE mutant probe for 20 mins in the presence of EcR common, EcR-B1 or EcR-A antibody. A mouse control antibody was also used. Complexes were resolved on an acrylamide/TBE gel, dried on 3MM Whatmann paper and exposed to Kodak film overnight. The EcR/Usp complex (EcR) and supershifted EcR/Usp complex (ss) are indicated. **(B)** Binding of the EcR complex was abolished upon mutation of the *dronc*EcRBE in nuclear extracts prepared from staged animals. EMSA analysis was carried out using isoform specific EcR antibodies. Mutation of the *dronc* EcRBE completely abolished an EcR-B1 supershifted complex. Times are in hours relative to puparium formation (h RPF).

A


Antibody or competitor
none
common
EcR-B1
EcR-A
control
hspEcRBE
none
hspEcRBE
common
ss
EcR/Usp
Probe
droncEcRBE
droncEcRBE mutant

B

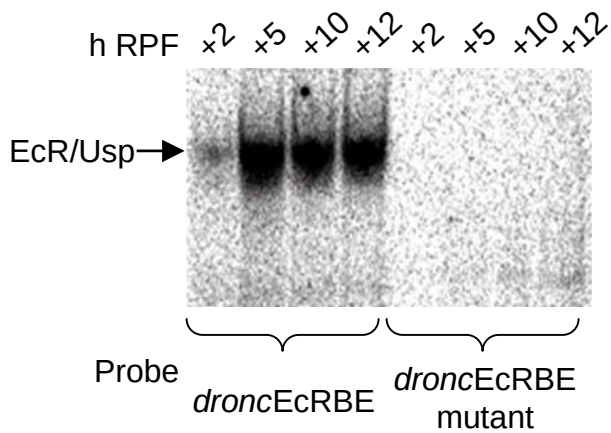
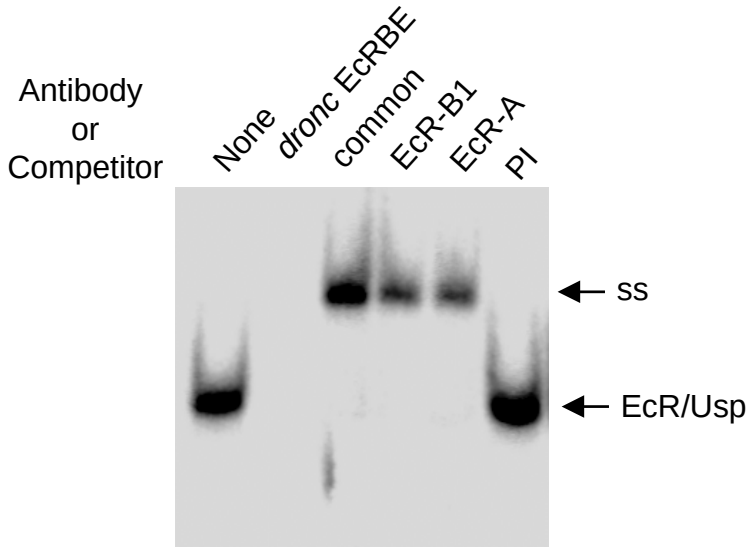

h RPF
+2
+5
+10
+12
+2
+5
+10
+12
EcR/Usp
Probe
droncEcRBE
droncEcRBE mutant

Figure 5.7 EcR-B1 binds the *dronc* promoter in the salivary glands and midgut.

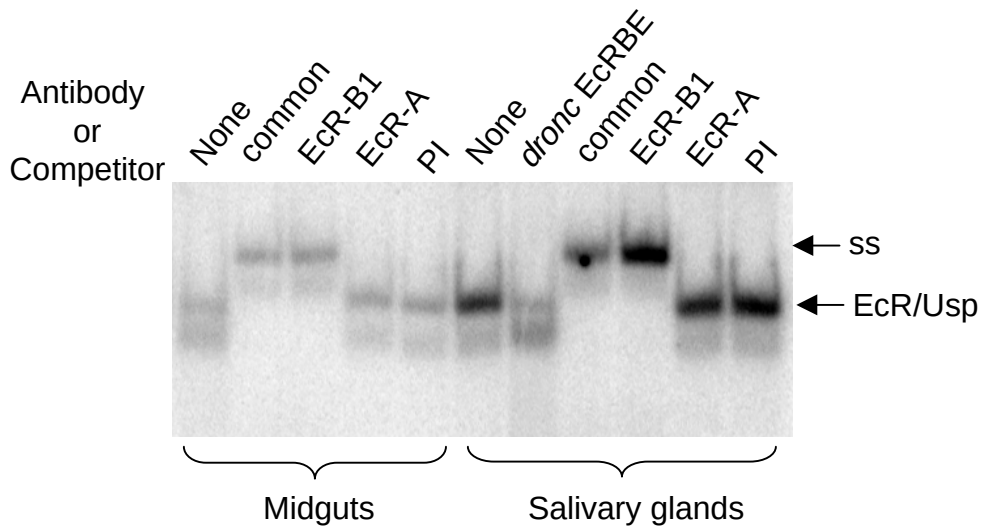
(A) EcR-B1 specific antibody can supershift a complex from mid prepupal nuclear extracts. **(B)** EcR-B1 specific antibody can supershift a complex from nuclear extracts from larval tissues prior to their destruction. Midgut samples are from early prepupal animals (+1 h RPF) and salivary glands are taken from early pupal (+12 h RPF) animals.

A



*dronc*EcRBE probe

B



B). These results demonstrate the EcR-B1 isoform specifically binds the *dronc* promoter *in vivo* at developmentally relevant times.

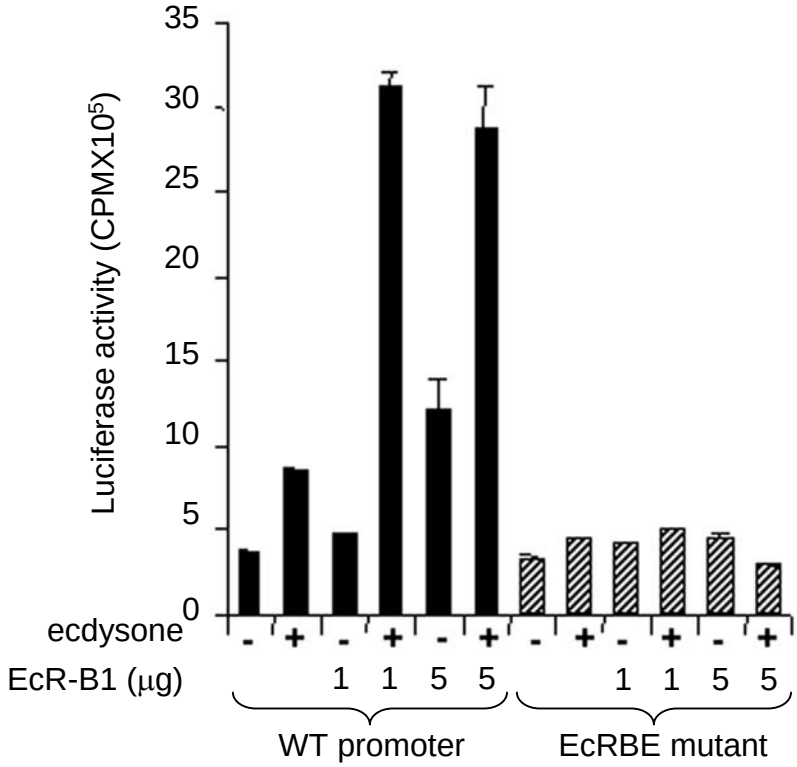
5.8 *droncEcRBE* is important for ecdysone-mediated *dronc* transcription

To establish a direct link between EcR-B1 isoform binding to the *dronc* promoter in response to ecdysone, *dronc* promoter-luciferase reporter constructs (with or without EcRBE mutations) were introduced into *l(2)mbn* cells and ecdysone-mediated reporter expression analysed. The WT *dronc* promoter was upregulated in response to ecdysone treatment and cotransfection of 1μg EcR-B1 isoform enhanced this ecdysone-mediated transcription (figure 5.8 A). Adding more EcR-B1 protein failed to enhance further the response to ecdysone, however, increasing EcR-B1 to 5μg raised the basal level of the luciferase reporter (figure 5.8 A). When the luciferase reporter had the EcRBE mutation introduced into its sequence, neither ecdysone nor increasing amounts of EcR-B1 protein could induce reporter activity. Since the region between 2.8–1.1 kb of the promoter recruits a putative histone deacetylase (HDAC) (D.Cakouros personal communication), Trichostatin A (TSA) was used to determine the role of EcRBE in mediating HDAC activity on the *dronc* promoter in the absence of ecdysone. The WT *dronc* promoter-reporter was activated by TSA treatment while the EcRBE mutation significantly inhibited this activation (figure 5.8 B). These results demonstrate that the EcR binding site possibly recruits a histone deacetylase for repression of transcription in the absence of ecdysone and is important for ecdysone-mediated *dronc* transcription.

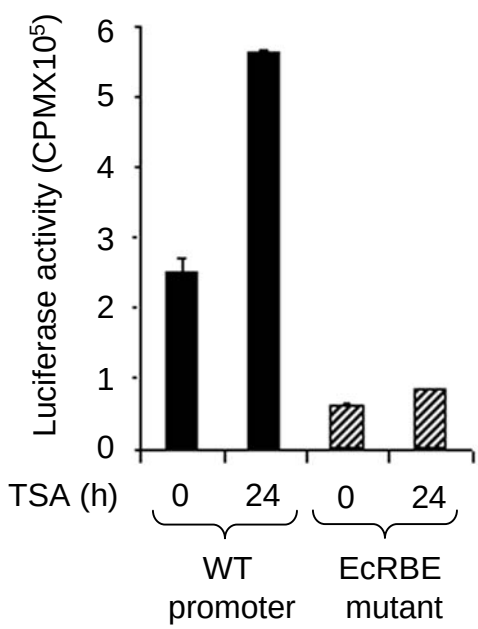
Figure 5.8 Mutation of *dronc* EcRBE reduces transcription.

(A) 2.5x10^6 *l(2)mbn* cells were transfected in triplicate with 2 mg of *dronc* luciferase reporter, under the control of the 2.8kb *dronc* promoter (WT) or the promoter with the EcRBE mutated (EcRBE mutant). Where indicated, cells were also cotransfected with 1 or 5 µg of the EcR-B1 expression vector. After 24 h cells were treated with 10 mM ecdysone for 24 h where indicated (+). Cell extracts were prepared and 100 mg protein assayed in triplicate for luciferase activity. Luciferase activity was subtracted from values obtained from empty luciferase vector transfections alone. **(B)** Transfections were as in (A) except Trichostatin A (TSA) was added at 1 mM as indicated. Error bars represent SD.

A


Luciferase activity (CPMX10^5)
35
30
25
20
15
10
5
0
ecdysone
- + - + - + - + - + - +
EcR-B1 (μg)
1 1 5 5 1 1 5 5
WT promoter
EcRBE mutant

B


Luciferase activity (CPMX10^5)
6
5
4
3
2
1
0
TSA (h)
0 24 0 24
WT promoter
EcRBE mutant

5.9 *dronc* EcRBE is important for tissue specific *dronc* expression

The ability of 2.8 kb of *dronc* promoter to correctly regulate the correct spatial and temporal expression of *dronc* was established in chapter 4 (Daish et al., 2003). It was also shown that deletion of the 2.8 kb promoter to 1.1 kb abolished expression in the larval salivary glands and brain lobes, but not in the midgut. The role of direct EcR-Usp-mediated *dronc* regulation was analysed in transgenic flies carrying mutations in the *dronc*EcRBE shown to prevent EcR-Usp binding. Transgenic flies carrying a *LacZ* reporter with a mutant EcRBE (in the 2.8kb *dronc* promoter *LacZ* transgene) were staged together with controls (WT 2.8kb-*LacZ dronc* promoter-reporter). Midguts and salivary glands were dissected and RNA extracted for Northern blotting to compare the effect of EcRBE mutation on reporter expression in tissues undergoing PCD *in vivo*. In WT *dronc* promoter lines, *LacZ* and endogenous *dronc* show low to undetectable levels of expression in midgut and salivary glands at late third instar (-12 h RPF) and +10 h RPF respectively. However, both *LacZ* and endogenous *dronc* transcription was upregulated in the midgut and salivary glands at +2 and +12 h RPF respectively (figure 5.9 A).

While mutation of the EcRBE did not significantly affect *LacZ* expression in the midgut, expression in the salivary gland was reduced at +12 h RPF compared to the control (figure 5.9 B). As expected, endogenous *dronc* was transcriptionally upregulated in both tissues at the expected times. These results demonstrate that the *dronc*EcRBE is required for the proper temporal expression of *dronc* in the salivary glands.

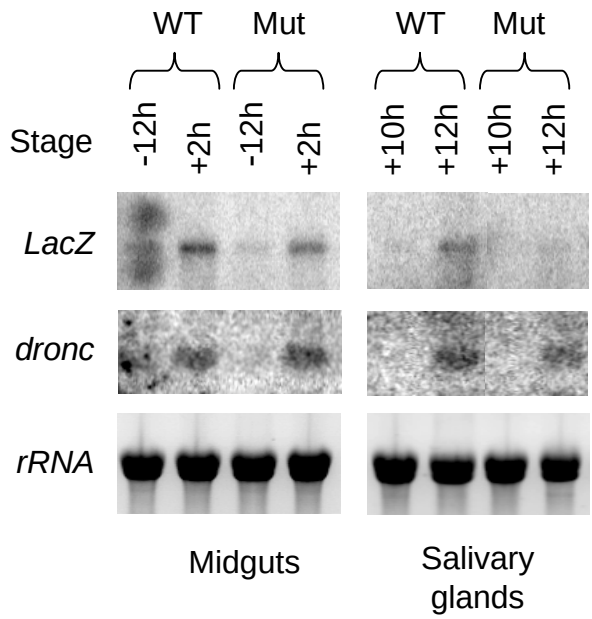


Figure 5.9 *dronc*EcRBE mutation disrupts tissue-specific temporal *dronc* expression.

RNA was collected from staged animals carrying the wild type *dronc* 2.8kb promoter-*LacZ* transgene (WT) or EcRBE mutant 2.8kb *dronc* promoter-*LacZ* transgene (Mut) at various times (in hours) relative to puparium formation from midguts (-12 and +2 h) and salivary glands (+10 and +12 h) and subjected to Northern blot analysis. Filters were probed with *LacZ* and *dronc*. A picture of the gel before blotting is shown to demonstrate that equal amounts of intact RNA were loaded in all lanes.

5.10 Discussion

The work presented in chapter 3 established that DRONC and its regulation play a critical role in development and that DRONC expression and function is tissue and stage specific. Chapter 4 demonstrated that correct *dronc* regulation requires specific promoter regions, and that these regions have specific dependencies on the hormone-induced transcription factors E93 and BR-C. These transcription factors are directly induced by the ecdysone receptor, as is the *rpr* promoter (Burtis et al., 1990; DiBello et al., 1991; Jiang et al., 2000; Karim and Thummel, 1992). Therefore to further link the hormone-induced transcriptional regulatory apparatus to DRONC function in development, investigation of the role of EcR in *dronc* transcriptional regulation is required.

One of the major questions associated with the biological actions of nuclear hormones is how a single hormone can control differentiation, PCD, and proliferation in different tissues. Deciphering how ecdysone mediates the spatial and temporal regulation of *dronc* expression will assist in understanding how hormones and nuclear receptors control PCD in specific tissues at precise stages of development.

The data in this chapter establishes that the promoter region spanning 2.8 kb to 1.1 kb contains important elements for ecdysone-mediated *dronc* transcription. It was shown that this region harbours a putative repressor that possibly acts by recruiting an HDAC. This is consistent with results in chapter 4 showing 1.1kb-*LacZ* activity to be much higher than 2.8kb-*LacZ* in specific tissues. Furthermore, the 1.1 kb-*LacZ* expression profile is temporally deregulated with expression being

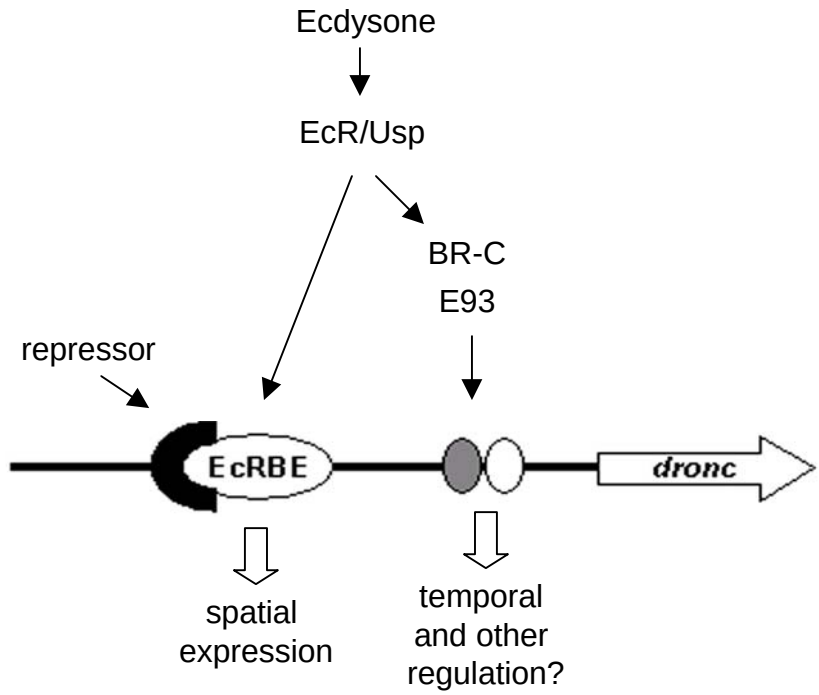


Figure 5.10 Model for the ecdysone-mediated regulation of *dronc* expression.

Ecdysone binds its heterodimeric receptor EcR/Usp and activates tissue-specific *dronc* expression by directly interacting with an EcRBE in the promoter. Recruitment of a potential repressor to the EcRBE region is likely to be required for the correct temporal regulation of *dronc* expression. EcR/Usp also regulates *dronc* transcription via the ecdysone-induced transcription factors BR-C and E93, which may also regulate temporal expression. Since E93 is required for expression of *dronc* in both larval salivary glands and midgut (Lee et al., 2000), it may also play a role in the spatial regulation of *dronc* expression.

maintained throughout the first and second larval instars when 2.8 kb-*LacZ* activity remains low.

An EcR/Usp binding site is present 1.36 kb from the *dronc* transcription start site and specifically binds the EcR-B1 isoform in *l(2)mbn* cells and *Drosophila* larval nuclear extracts. This is consistent with EcR isoform expression profiles demonstrating a positive correlation between EcR-B1 and cell death regulation as it appears in tissues destined to undergo PCD (Talbot et al., 1993; Truman et al., 1994). Functional experiments in *l(2)mbn* cells established that this site is important for *dronc* transcription as mutation of the binding consensus sequence reduces ecdysone-mediated activation and transactivation by EcR-B1. The *l(2)mbn* cell line is derived from haemocytes, which, as discussed in chapter 3, have an active DRONC-dependent death programme evidenced by *dronc*^{51} blood cells being resistant to multiple death stimuli (Chew et al., 2004). Results gained using this *ex vivo* cell culture system provide physiologically relevant predictions for cell death mechanisms occurring *in vivo*.

In vivo evidence of the importance of the identified *dronc*EcRBE consensus is supported by transgenic promoter-reporter analyses showing deletion of the sequence containing the EcRBE abrogated salivary gland reporter expression (figure 5.3 A). However, mutation of the EcRBE in the 2.8 kb-*LacZ* transgene delayed induction in the salivary glands. This indicates that correct temporal timing of *dronc* expression in the salivary glands is EcR-B1-dependent but transcriptional activity still occurs, although delayed, due to elements contained within the 1.33-2.8 kb and proximal promoter such as BR-C Z1 binding sites (Cakouros et al., 2002). As 2.8 kb

confers endogenous activity, 1.64-2.8 kb must contain elements modulating expression in the salivary glands. Experiments are currently being conducted in our laboratory to identify E93 binding sites on the *dronc* promoter.

Promoter deletion to 1.33 kb had no qualitative effect on reporter expression in the midgut. The *dronc* promoter reporter analyses above and in chapter 4 indicate that the transcriptional environments of the midgut and salivary glands permit access to, or require, different minimal promoter regions to effect transcriptional activity in the midgut. This is demonstrated not only by qualitative but also quantitative differences between the transcriptional responses of the same transgene occurring between the two tissues as observed for the 1.1 kb-*LacZ* activity profile. As well as the *BR-C* and *E93* mutant promoter reporter studies, the EcRBE mutation data extends the understanding of such differential regulatory requirements between these tissues. Such differences may also be due to different chromatin structures forming along the *dronc* promoter between these tissues or more specifically, the chromatin structure immediately surrounding the EcR binding site. In the midgut this may preclude EcR/Usp binding to the EcRBE. The presence of, as yet unidentified, midgut-specific repressors may be preventing an EcR complex from binding the *dronc* promoter in this tissue at specific developmental times.

Tissue specific differences in intracellular environments were obviated by the *dronc* mutant phenotype data in chapter 3 describing the loss of DRONC as having a markedly different impact on the destruction of the midguts compared to the salivary glands. The cell death transcriptional hierarchy acting in the midgut and salivary glands differs at the various levels of regulation investigated in this thesis. Firstly, the

ability of the EcR/Usp to effect correct hormone-induced transcription in the midgut and salivary glands differs in regard to the temporal expression profile of *dronc*. Secondly, the BR-C and E93 transcription factors differ in their death signal transducing ability between these tissues in regard to inducing core death gene transcription. Thirdly, DRONC function differs between the midgut and salivary glands in regard to effector caspase activation and its involvement in causing apoptotic phenotypes such as DNA fragmentation, vacuolar dynamics, and ultrastructural changes.

The EcR-B1 isoform is directly recruited to the *dronc* promoter and is essential for correct tissue specific *dronc* transcription, the control of which also depends on BR-C and E93. The function of these transcription factors also depends on the tissue-specific context. The data presented in this chapter thus forms the foundation of future work to address questions associated with tissue specific gene expression and PCD and the role of nuclear hormones in controlling these processes.

Experimental priorities include elucidation of the role of E93 in the transcriptional control of *dronc*. The *in vitro* production of either full length or the DNA binding region of E93 protein to use in DNA footprinting along the *dronc* promoter will identify functional E93 DNA binding consensus sequences. Identification of general promoter regions critical in E93-mediated *dronc* expression can also be approached by employing salivary gland and midgut specific GAL4 driven E93 transgenes in conjunction with *dronc* promoter-reporter transgenes such as those used in chapter 4 and above. Furthermore, *in vivo* analysis of the BR-C Z1 proximal binding consensus identified by Cakouros and colleagues (2002) will aid in the formation of a

transcription regulation model describing the cooperative roles of BR-C, E93, and the EcR/Usp complex on the *dronc* promoter in the salivary glands and midgut.

Chapter 6

General Discussion

General Discussion

When beginning the work presented in this thesis, little direct *in vivo* evidence existed to demonstrate an essential requirement for DRONC for cell deaths throughout development or for the successful completion of development itself. Furthermore, despite the fact that *dronc* expression was tightly regulated during development, no promoter analyses of *dronc* transcriptional regulation by ecdysone had been undertaken. Ectopic expression of *dronc* and embryo dsRNA injections were largely the techniques employed to show that DRONC was able to induce apoptosis in a living animal and was also required for cell deaths during embryogenesis (Dorstyn et al., 1999a; Quinn et al., 2000). Additionally, caspase mutants in the fly had not been analysed and the concept of transcriptional control of core death genes in the regulation of developmental cell deaths was poorly understood. The paradigm that caspases were involved in physiological processes other than programmed cell death had not been firmly established. The generation of libraries of P-elements saturating the *Drosophila* genome has made hypomorphic mutant acquisition and the excision of such elements to generate null mutants of many novel target genes a more experimentally amenable task.

Work by Chew and colleagues (2004), done in parallel with that presented in this thesis, provide additional and complementary data on the role of DRONC in development. This work, and the work in chapter 3 are co-published in the December issue of Developmental Cell. These investigators generated a *dronc* mutant by the excision of the *KGO2994* P-element. Their legion, named *dronc*^{51}, differed to that

described in chapter 3 in that it was unidirectional and did not excise the *CG6685* gene flanking *dronc*. Embryos from females heterozygous for the hypomorphic *KGO2994* allele and the *dronc*^{51} chromosome fail to hatch and have a deficit in embryonic cell deaths resulting in a *hid* mutant phenotype (Chew et al., 2004; Grether et al., 1995). *dronc*^{51} animals were late larval lethal, had slightly enlarged CNS and imaginal discs, and blood cell hyperplasia. Clonal analysis demonstrated limited impact of *dronc* mutation on eye or wing development. These data have two significant differences to the *dronc*^{d5} phenotype. Firstly, *dronc*^{d5} larvae have no comparable larval organ enlargement and secondly, *dronc*^{d5} lethality is much later in development permitting analysis of midgut and salivary gland cell death.

Maternal *dronc* is required for developmental progression as *dronc*^{d5} animals, which have the maternal *dronc* contribution from their heterozygous mother, reach the pupal stage before obvious gross morphological defects are observed (Daish et al., 2004). However, these animals do have deficits in imaginal disc cell deaths and persistence of the haemocyte population. In regard to the former, one would expect less cell death to result in aberrant organ formation as observed in *dronc*^{51} larval tissues, however, detailed size and cell number analyses of *dronc*^{d5} wing discs failed to detect significant differences to the WT structure. The possibility that haemocyte hyperplasia contributes to *dronc*^{51} larval lethality can be precluded as *dronc*^{d5} animals also exhibit this phenotype and have a pupation frequency of 100%. Experiments are currently being undertaken to determine the causation of blood cell persistence, be it lack of cell death or excess proliferation, and the specific cell types that persist. Also the response to ecdysone of *dronc*^{d5} blood cells cultured *ex vivo* is also in progress.

This is of particular interest in regard to determining mechanisms of effector caspase activation in the absence of DRONC.

Established dogma predicts DRONC to be the primary molecule to activate effector caspases *in vivo* due to it being the only CED-3/caspase-9 homologue in the fly (Dorstyn et al., 1999a; Salvesen and Abrams, 2004). Chapter 3 details the novel finding that effector caspase activation can occur in the midgut in the absence of DRONC, while it is dependent on DRONC in the salivary glands. The mechanism by which caspases are activated in the midgut without DRONC remains to be clarified, but whatever is transducing the death signal appears to be active well before the time of *dronc* upregulation, as high caspase substrate cleavage levels are observed in midguts of third instar WT and *dronc*^{d5} larvae. A suppressor screen in the *dronc*^{d5} genetic background may be a valid strategy to identify DRONC-independent mediators of the death-signalling pathway in the midgut. Scoring aspects of midgut persistence may be problematic, however it could be assayed by caspase activity. Midgut caspase activity and destruction should be analysed in *dark* mutants as DARK may be an adaptor protein required to activate caspases independent of DRONC. In such a case *dark/dronc* double mutants should have reduced caspase activity. However, if a mechanism independent of DARK/DRONC activates effector caspases then *dark* mutant midguts should undergo apoptosis as in *dronc*^{d5} animals.

To resolve the question as to what is causing the *dronc*^{d5} lethality, a number of experimental strategies may be employed. If persistence of the larval salivary glands prevents normal pupation, the following experiments are warranted to confirm this; (i) Under the control of the salivary gland specific forkhead-GAL4 driver, UAS-*dronc*

transgene expression in *dronc*^{d5} salivary glands would induce death and cause removal of this tissue, which will determine whether developmental abnormalities present in *dronc*^{d5} animals, other than salivary gland persistence, are responsible for the pupal lethality. Premature salivary gland specific *dronc* expression, as previously tested in our laboratory, leads to destruction of the tissue. (ii) The same forkhead-GAL4 driver expressing a dominant negative form of DRONC in the salivary glands of WT animals, previously shown to cause a failure of salivary gland removal in 10% of animals (Martin and Baehrecke, 2004), could be used to test whether salivary gland persistence affects viability throughout pupation. To identify animals with persistent glands without dissection, a UAS-GFP transgene could be expressed in the salivary glands, also driven by the forkhead-GAL4 driver. (iii) The FLP/FRT system could be used to generate *dronc*^{d5} clones specifically in the forming salivary glands so only the cells in this tissue have the *dronc*^{d5} or mutant genotype in an otherwise WT animal. This would establish whether salivary gland persistence is linked to the causation of *dronc*^{d5} pupal lethality.

The importance of the *CG6685* gene in development was shown in chapter 3, however, a definitive demonstration that the temporal and spatial distributions of CG6685 protein is restored in *dronc*^{d5} animals could not be undertaken due to the non-availability of appropriate reagents. The embryonic expression pattern should be analysed by *in situ* hybridisation to see if this gene is expressed in specific cell populations and may shed light on its function in development. These experiments are currently being undertaken and will further validate the phenotype of *dronc*^{d5} animals being solely attributed to mutation of *dronc*. Regarding the *dronc*^{51} mutation

generated by Chew and colleagues (2004), this is of particular relevance due to the possibility of some *CG6685* dysregulation, something that *in situ* hybridisation and immunohistochemical analysis would clarify. *dronc*^{51} lethality was rescued to eclosion when the *dronc* rescue transgene described in chapter 3 was crossed into *dronc*^{51} animals. While this rescue occurred at a low frequency, it does suggest that any *CG6685* dysregulation has a minimal contribution to the *dronc*^{51} phenotype. Inconsistencies between the *dronc*^{51} and *dronc*^{d5} mutant phenotypes may be reconciled by clarification of CG6685 function and the consequence, if any, of removing its promoter sequence through *dronc* excision. To gain some understanding of the function of CG6685, I have generated *UAS-CG6685* fly lines and expressed this transgene in the larval eye disc. No obvious gross morphological defect was observed, however, only single copies of the GAL4 driver and *UAS-CG6685* transgenes were used (Daish, Mills and Kumar, unpublished data).

More evidence is emerging supporting a role for caspases in diverse physiological processes. In mammals, some caspases are involved in maintenance of lymphocyte homeostasis, but they are also required for the proliferation of primary human T-cells, with caspase-8 specifically shown to be involved in this process (Alam et al., 1999; Chun et al., 2002; Kennedy et al., 1999). Cell cycle regulators like Wee1 have been identified as caspase cleavage targets (Alam et al., 1999; Zhou et al., 1998). Differentiation of blood cells during erythropoiesis and keratinocyte formation also have been shown to be caspase-dependent, the former involving caspase-3 and –9 activation and the latter specifically involving caspase-14 (De Botton et al., 2002; Eckhart et al., 2000; Schwerk and Schulze-Osthoff, 2003). Caspase-3 and Apaf-1 are

required for the physiological death of granulosa cells surrounding the murine oocyte during follicle regression, however, the germ cell environment in *Drosophila* appears somewhat privileged in that DIAP1 removal does not necessarily result in effector caspase activation (Geisbrecht and Montell, 2004; Robles et al., 1999). Thus the *Drosophila* egg chamber may provide insights into alternative mechanisms of caspase regulation. Recently, caspase-8 was shown to be required for normal endothelial cell function which, in caspase-8 mutants, leads to circulatory failures involving cardiac related abnormalities (Kang et al., 2004). As well as defects in haemopoietic progenitor cell function, macrophage precursor differentiation had a significant caspase-8 dependence (Kang et al., 2004). Caspase-3 knockout mice show defects in skeletal muscle fibre cell differentiation possibly due to disruption of actin fibre reorganisation (Fernando et al., 2002; Schwerk and Schulze-Osthoff, 2003). Thus a key question to be answered is what cellular mechanisms determine how a cell can utilise aspects of caspase function while preventing cell death, and how this occurs in the context of normal development and tissue formation.

The GTPase Rac was recently shown to be required for the migration of anterior epithelial cells through the nurse cell population of the egg chamber follicle to the oocyte/nurse cell interface during oogenesis (Geisbrecht and Montell, 2004). A screen to identify suppressors of a dominant negative (RacN17) Rac-induced border cell migration defect showed DIAP1 overexpression was able to suppress the phenotype, as did mutation of *dark*. This demonstrates that inhibition of DRONC activation, either by increasing DIAP1 or by removal of functional DARK protein, restores normal border cell migration. Furthermore, loss of DIAP1 function, which in

other tissues causes apoptosis by deregulation of caspase activation, results in border cell migration defects but no apoptosis. To investigate this process by analysing egg chambers from females heterozygous for the *KGO2994* allele and the *dronc*^{d5} chromosome (*dronc* hypomorphs) may not reveal DRONCs role in border cell migration under normal physiological conditions as this requires repression of DRONC action.

Recently, multiple research groups have been exploiting the *Drosophila* wing disc to explore caspase function in non-apoptotic events (Huh et al., 2004a; Perez-Garijo et al., 2004; Ryoo et al., 2004). In response to excessive cell deaths, wing discs activate compensatory proliferation mechanisms to restore normal wing size and structure (Milan, 1998; Neufeld et al., 1998). Recent investigators hypothesised that this process was linked to the cell death event and possibly to the molecules controlling death (Huh et al., 2004a; Perez-Garijo et al., 2004; Ryoo et al., 2004). By inducing cell death signalling by *hid* expression, but blocking cell death execution with p35 expression, increased proliferation due to ectopic expression of the mitogen Wingless was observed, and importantly, this was dependent on the presence of the active form of DRONC (Huh et al., 2004a). One may hypothesise that in the absence of DRONC there should be less cell death and a consequent reduction in proliferative signals from dying cells. This would result in less cell division and thus maintenance of the total cell complement. However, larval wing discs from *dronc*^{d5} animals had no obvious reductions in BrdU staining. This is probably because in normal wing disc development there is minimal apoptosis and therefore detectable compensatory mechanisms involving DRONC may be utilised only when normal development is

disrupted. Perez-Garijo et al. (2004) achieved this by X-ray and heat shock exposure while Huh et al. (2004) used *hid* expression. In the process of the limited physiological cell deaths in the wing disc, DRONC most likely serves a dual function. The death signalling pathway splits at the DRONC activation event, presumably involving DARK, one DRONC target is the effector caspase DRICE leading to apoptosis, the other target is an unknown messenger which signals Wg to induce cell division to replace the dying cell. Ryoo and colleagues (2004) provide a compensatory proliferation model whereby DIAP1 plays a central role in blocking the signals, which activate the JNK pathway and lead to *dpp* and *wg* upregulation. Apoptotic stimuli cause the RPR and HID death activators to relieve DIAP1 repression on DRONC and the effector caspases, and also TRAF1 which initiates JNK signalling (Ryoo et al., 2004).

To explore the role of DRONC in compensatory proliferation in the wing disc, the cell death signalling pathway could be induced in *dronc*^{d5} animals by X-ray exposure, however a hypothesis on the outcome is hard to formulate for the following reasons. Inducing death signalling in the wing results in a normal adult wing due to considerable compensatory proliferation. Blocking caspase activation in the wing disc does not prevent normal wing formation (Perez-Garijo et al., 2004). Due to limited apoptosis occurring in the normal unstimulated wing disc, compensatory mechanisms involving proliferation may be difficult to detect in *dronc*^{d5} unstimulated wing discs. *dronc*^{d5} wing discs do not have increased cell deaths in response to radiation exposure (chapter 3). Therefore, not only is there no apoptosis to compensate for, but the molecule on which the Wg-mediated compensatory proliferation is dependent, DRONC, is also absent. However, the activity of *Wg* and *Dpp* could be tested in such

wing discs for their dependence on DRONC in death-stimulated cells, which are death inhibited.

The *in vitro* and *in vivo dronc* promoter analyses in chapters 3 and 4 and by Cakouros et al. (2002) show that *dronc* transcriptional regulation is complex and involves the cooperative binding of the BR-C and E93 transcription factors and the EcR/Usp nuclear receptor heterodimer. These factors all use distinct regions of the *dronc* promoter in a stage and tissue specific manner to control either the temporal, spatial or quantitative aspects of *dronc* expression. However, the following key areas of *dronc* regulation are yet to be resolved.

Cakouros et al. (2002) showed BR-C Z1 specific complexes forming on the *dronc* proximal promoter at developmental times preceding ecdysone-induced *dronc* upregulation. Similar complexes were observed to form on the *dronc* proximal promoter in *l(2)mbn* cells following ecdysone exposure. While two 60 bp *dronc* promoter fragments (D4; -7 to –66 and D6; -117 to -176) were differentially competed by multiple cold BR-C isoform consensus oligos, this does not demonstrate definitively isoform specific binding. However, BR-C Z1 specific complexes were competed out from late larval and prepupal nuclear extracts, and BR-C Z1 protein was able to transactivate a *dronc* proximal promoter luciferase reporter construct. To better understand how BR-C isoforms regulate *dronc* transcription in the midgut and salivary glands, EMSA analysis using D4 sequence and binding BR-C isoform consensus cold competitors in conjunction with BR-C isoform specific antibodies, must be used with prepupal midgut and +12 h RPF salivary gland nuclear extracts.

This will determine the precise promoter requirements regarding BR-C isoform binding at the *dronc* proximal promoter.

2.8 kb of *dronc* promoter was not transcriptionally active in the midguts or salivary glands of *E93* mutant animals at the time of destruction of these tissues. This is consistent with the impact on endogenous *dronc* in *E93* mutant animals (Lee et al., 2002a; Lee et al., 2002b; Lee et al., 2000). Thus, while the BR-C Z1 appears to mainly function in the salivary glands to induce *dronc* expression, E93 appears to be a critical regulator in both tissues. Therefore the binding profile of E93 on the *dronc* promoter must be elucidated as outlined in the chapter 5 discussion. Employment of the E93 antibody in conjunction with ChiP analyses along the *dronc* promoter would address these questions. It is predicted that E93 acts cooperatively with BR-C Z1 to regulated stage-specific *dronc* expression in the salivary glands. Why mutation of the EcR/Usp binding site does not prevent the eventual promoter reporter expression in the salivary glands, may also be better understood following these experiments.

The results presented in this thesis describe the first characterisation of a mutation in the apical caspase *dronc* and the first functional dissection of the *dronc* promoter. These studies revealed that DRONC is essential for early development, is required for many physiological and stress-induced cell deaths, and for the destruction of the larval salivary glands. Significantly, it is shown that the dependence of effector caspase activation on DRONC is a tissue-dependent relationship. The genetics employed to generate the *dronc* specific mutation makes it a unique model system which can now be exploited to further investigate the role of DRONC in development and its function in the context of non-apoptotic processes occurring in

the gonads or the recently exploited model tissue, the imaginal wing disc. Furthermore, *ex vivo* culture of persistent *dronc*^{d5} haemocytes provides another valuable model system to study DRONC-independent hormone-induced caspase activation mechanisms. *dronc* has also been established as an ideal model gene for studying mechanisms of transcription due to its restricted expression profile. Greater understanding of gene regulation during programmed cell death lays the foundation for the formulation of a more complete and detailed model of hormone-induced and nuclear receptor-mediated cell death regulation.

Abrams, J. M., White, K., Fessler, L. I., and Steller, H. (1993). Programmed cell death during Drosophila embryogenesis. Development *117*, 29-43.

Alam, A., Cohen, L. Y., Aouad, S., and Sekaly, R. P. (1999). Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. J Exp Med *190*, 1879-1890.

Antoniewski, C., Mugat, B., Delbac, F., and Lepesant, J. A. (1996). Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in Drosophila melanogaster. Mol Cell Biol *16*, 2977-2986.

Ashburner, M. (1967). Patterns of puffing activity in the salivary gland chromosomes of Drosophila. I. Autosomal puffing patterns in a laboratory stock of Drosophila melanogaster. Chromosoma *21*, 398-428.

Ashburner, M., and Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster. III. Consequences of ecdysone withdrawal. Dev Biol *54*, 241-255.

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

Baehrecke, E. H. (2002). How death shapes life during development. Nat Rev Mol Cell Biol *3*, 779-787.

Baehrecke, E. H., and Thummel, C. S. (1995). The Drosophila E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. Dev Biol *171*, 85-97.

Bayer, C. A., Holley, B., and Fristrom, J. W. (1996). A switch in broad-complex zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of Drosophila imaginal discs. Dev Biol *177*, 1-14.

Bayer, C. A., von Kalm, L., and Fristrom, J. W. (1997). Relationships between protein isoforms and genetic functions demonstrate functional redundancy at the Broad-Complex during Drosophila metamorphosis. Dev Biol *187*, 267-282.

Belyaeva, E. S., Aizenzon, M. G., Semeshin, V. F., Kiss, II, Koczka, K., Baritcheva, E. M., Gorelova, T. D., and Zhimulev, I. F. (1980). Cytogenetic analysis of the 2B3-4--2B11 region of the X-chromosome of Drosophila melanogaster. I. Cytology of the region and mutant complementation groups. Chromosoma *81*, 281-306.

Bender, M., Imam, F. B., Talbot, W. S., Ganetzky, B., and Hogness, D. S. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. Cell *91*, 777-788.

Bickler, P. E., and Donohoe, P. H. (2002). Adaptive responses of vertebrate neurons to hypoxia. J Exp Biol *205*, 3579-3586.

Bodenstein, D. (1965). The postembryonic development of *Drosophila*. In Biology of *Drosophila*, M. Demerec, ed. (New York, Hafner Publishing Company), pp. 275-267.

Bolton, D. L., Hahn, B. I., Park, E. A., Lehnhoff, L. L., Hornung, F., and Lenardo, M. J. (2002). Death of CD4(+) T-cell lines caused by human immunodeficiency virus type 1 does not depend on caspases or apoptosis. J Virol *76*, 5094-5107.

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.

Bratton, S. B., and Cohen, G. M. (2003). Death receptors leave a caspase footprint that Smacs of XIAP. Cell Death Differ *10*, 4-6.

Broadus, J., McCabe, J. R., Endrizzi, B., Thummel, C. S., and Woodard, C. T. (1999). The Drosophila beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol Cell *3*, 143-149.

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.

Brodu, V., Mugat, B., Roignant, J. Y., Lepesant, J. A., and Antoniewski, C. (1999). Dual requirement for the EcR/USP nuclear receptor and the dGATAb factor in an ecdysone response in Drosophila melanogaster. Mol Cell Biol *19*, 5732-5742.

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.

Cakouros, D., Daish, T., Martin, D., Baehrecke, E. H., and Kumar, S. (2002). Ecdysone-induced expression of the caspase DRONC during hormone-dependent programmed cell death in Drosophila is regulated by Broad-Complex. J Cell Biol *157*, 985-995.

Cakouros, D., Daish, T. J., and Kumar, S. (2004). Ecdysone receptor directly binds the promoter of the Drosophila caspase dronc, regulating its expression in specific tissues. J Cell Biol *165*, 631-640.

Campos, A. R., Grossman, D., and White, K. (1985). Mutant alleles at the locus elav in Drosophila melanogaster lead to nervous system defects. A developmental-genetic analysis. J Neurogenet *2*, 197-218.

Carney, G. E., and Bender, M. (2000). The Drosophila ecdysone receptor (EcR) gene is required maternally for normal oogenesis. Genetics *154*, 1203-1211.

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.

Chai, J., Yan, N., Huh, J. R., Wu, J. W., Li, W., Hay, B. A., and Shi, Y. (2003). Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. Nat Struct Biol *10*, 892-898.

Chao, S., and Nagoshi, R. N. (1999). Induction of apoptosis in the germline and follicle layer of Drosophila egg chambers. Mech Dev *88*, 159-172.

Chen, P., Nordstrom, W., 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 reaper, grim, and hid in Drosophila. Dev Biol *201*, 202-216.

Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001). BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell *8*, 705-711.

Chew, S. K., Akdemir, F., Chen, P., Lu, W. J., Mills, K., Daish, T., Kumar, S., Rodriguez, A., and Abrams, J. M. (2004). The Apical Caspase dronc Governs Programmed and Unprogrammed Cell Death in Drosophila. Dev Cell *7*, 897-907.

Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V., and Dixit, V. M. (1997a). Role of CED-4 in the activation of CED-3. Nature *388*, 728-729.

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

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S. I., and Abrams, J. M. (2002). The damage-responsive Drosophila gene sickle encodes a novel IAP binding protein similar to but distinct from reaper, grim, and hid. Curr Biol *12*, 137-140.

Chun, H. J., Zheng, L., Ahmad, M., Wang, J., Speirs, C. K., Siegel, R. M., Dale, J. K., Puck, J., Davis, J., Hall, C. G.*, et al.* (2002). Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. Nature *419*, 395-399.

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.

Crispi, S., Giordano, E., D'Avino, P. P., and Furia, M. (1998). Cross-talking among Drosophila nuclear receptors at the promiscuous response element of the ng-1 and ng-2 intermolt genes. J Mol Biol *275*, 561-574.

Crispi, S., Giordano, E., D'Avino, P. P., Peluso, I., and Furia, M. (2001). Functional analysis of regulatory elements controlling the expression of the ecdysone-regulated Drosophila ng-1 gene. Mech Dev *100*, 25-35.

Crossgrove, K., Bayer, C. A., Fristrom, J. W., and Guild, G. M. (1996). The Drosophila Broad-Complex early gene directly regulates late gene transcription during the ecdysone-induced puffing cascade. Dev Biol *180*, 745-758.

Cryns, V., and Yuan, J. (1998). Proteases to die for. Genes Dev *12*, 1551-1570.

Daish, T. J., Cakouros, D., and Kumar, S. (2003). Distinct promoter regions regulate spatial and temporal expression of the Drosophila caspase dronc. Cell Death Differ *10*, 1348-1356.

Daish, T. J., Mills, K., and Kumar, S. (2004). Drosophila Caspase DRONC Is Required for Specific Developmental Cell Death Pathways and Stress-Induced Apoptosis. Dev Cell *7*, 909-915.

D'Avino, P. P., Crispi, S., Polito, L. C., and Furia, M. (1995). The role of the BR-C locus on the expression of genes located at the ecdysone-regulated 3C puff of Drosophila melanogaster. Mech Dev *49*, 161-171.

De Botton, S., Sabri, S., Daugas, E., Zermati, Y., Guidotti, J. E., Hermine, O., Kroemer, G., Vainchenker, W., and Debili, N. (2002). Platelet formation is the consequence of caspase activation within megakaryocytes. Blood *100*, 1310-1317.

Degterev, A., Boyce, M., and Yuan, J. (2003). A decade of caspases. Oncogene *22*, 8543-8567.

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.

Donepudi, M., Mac Sweeney, A., Briand, C., and Grutter, M. G. (2003). Insights into the regulatory mechanism for caspase-8 activation. Mol Cell *11*, 543-549.

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

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

Doumanis, J. (2004) Characterisation of a novel caspase STRICA and the Bcl-2 homologues BUFFY and DEBCL in *Drosophila melanogaster*.

Doumanis, J., Quinn, L., 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 *8*, 387-394.

Dubrovsky, E. B., Dretzen, G., and Bellard, M. (1994). The Drosophila broad-complex regulates developmental changes in transcription and chromatin structure of the 67B heat-shock gene cluster. J Mol Biol *241*, 353-362.

Dubrovsky, E. B., Dretzen, G., and Berger, E. M. (1996). The Broad-Complex gene is a tissue-specific modulator of the ecdysone response of the Drosophila hsp23 gene. Mol Cell Biol *16*, 6542-6552.

Dubrovsky, E. B., Dubrovskaya, V. A., and Berger, E. M. (2001). Selective binding of Drosophila BR-C isoforms to a distal regulatory element in the hsp23 promoter. Insect Biochem Mol Biol *31*, 1231-1239.

Duffy, J. B. (2002). GAL4 System in *Drosophila*: A Fly Geneticist's Swiss Army Knife. Genesis *34*, 1-15.

Eckhart, L., Declercq, W., Ban, J., Rendl, M., Lengauer, B., Mayer, C., Lippens, S., Vandenabeele, P., and Tschachler, E. (2000). Terminal differentiation of human keratinocytes and stratum corneum formation is associated with caspase-14 activation. J Invest Dermatol *115*, 1148-1151.

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

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

Emery, I. F., Bedian, V., and Guild, G. M. (1994). Differential expression of Broad-Complex transcription factors may forecast tissue-specific developmental fates during Drosophila metamorphosis. Development *120*, 3275-3287.

Fearnhead, H. O. (2004). Getting back on track, or what to do when apoptosis is de-railed: recoupling oncogenes to the apoptotic machinery. Cancer Biol Ther *3*, 21-28.

Fernando, P., Kelly, J. F., Balazsi, K., Slack, R. S., and Megeney, L. A. (2002). Caspase 3 activity is required for skeletal muscle differentiation. Proc Natl Acad Sci U S A *99*, 11025-11030.

Fletcher, J. C., Burtis, K. C., Hogness, D. S., and Thummel, C. S. (1995). The Drosophila E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to ecdysone. Development *121*, 1455-1465.

Fletcher, J. C., and Thummel, C. S. (1995a). The Drosophila E74 gene is required for the proper stage- and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. Development *121*, 1411-1421.

Fletcher, J. C., and Thummel, C. S. (1995b). The ecdysone-inducible Broad-complex and E74 early genes interact to regulate target gene transcription and Drosophila metamorphosis. Genetics *141*, 1025-1035.

Foley, K., and Cooley, L. (1998). Apoptosis in late stage Drosophila nurse cells does not require genes within the H99 deficiency. Development *125*, 1075-1082.

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

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.

Geisbrecht, E. R., and Montell, D. J. (2004). A role for Drosophila IAP1-mediated caspase inhibition in Rac-dependent cell migration. Cell *118*, 111-125.

Goyal, L. (2001). Cell death inhibition: keeping caspases in check. Cell *104*, 805-808.

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

Guay, P. S., and Guild, G. M. (1991). The ecdysone-induced puffing cascade in Drosophila salivary glands: a Broad-Complex early gene regulates intermolt and late gene transcription. Genetics *129*, 169-175.

Hall, B. L., and Thummel, C. S. (1998). The RXR homolog ultraspiracle is an essential component of the Drosophila ecdysone receptor. Development *125*, 4709-4717.

Harlin, H., Reffey, S. B., Duckett, C. S., Lindsten, T., and Thompson, C. B. (2001). Characterization of XIAP-deficient mice. Mol Cell Biol *21*, 3604-3608.

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 *276*, 25342-25350.

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., 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.

Hazelrigg, T. (2000). In Drosophila Protocols, W. Sullivan, Ashburner, M., and Hawley, R. S., ed. (Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press), pp. 313-343.

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

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

Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999). Phylogenetic perspectives in innate immunity. Science *284*, 1313-1318.

Horner, M. A., Chen, T., and Thummel, C. S. (1995). Ecdysteroid regulation and DNA binding properties of Drosophila nuclear hormone receptor superfamily members. Dev Biol *168*, 490-502.

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

Huh, J. R., Guo, M., and Hay, B. A. (2004a). Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. Curr Biol *14*, 1262-1266.

Huh, J. R., Vernooy, S. Y., Yu, H., Yan, N., Shi, Y., Guo, M., and Hay, B. A. (2004b). Multiple apoptotic caspase cascades are required in nonapoptotic roles for Drosophila spermatid individualization. PLoS Biol *2*, E15.

Jacobson, M. D., Weil, M., and Raff, M. C. (1997). Programmed cell death in animal development. Cell *88*, 347-354.

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

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

Kang, T. B., Ben-Moshe, T., Varfolomeev, E. E., Pewzner-Jung, Y., Yogev, N., Jurewicz, A., Waisman, A., Brenner, O., Haffner, R., Gustafsson, E.*, et al.* (2004). Caspase-8 serves both apoptotic and nonapoptotic roles. J Immunol *173*, 2976-2984.

Karim, F. D., Guild, G. M., and Thummel, C. S. (1993). The Drosophila Broad-Complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development *118*, 977-988.

Karim, F. D., and Thummel, C. S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. Embo J *11*, 4083-4093.

Kennedy, N. J., Kataoka, T., Tschopp, J., and Budd, R. C. (1999). Caspase activation is required for T cell proliferation. J Exp Med *190*, 1891-1896.

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

Kiss, I., Beaton, A. H., Tardiff, J., Fristrom, D., and Fristrom, J. W. (1988). Interactions and developmental effects of mutations in the Broad-Complex of Drosophila melanogaster. Genetics *118*, 247-259.

Kiss, I., Szabad, J., Belyaeva, E. S., Zhimulev, I. F., and Major, J. (1980). Genetic and developmental analysis of mutants in an early ecdysone-inducible puffing region in Drosophila melanogaster. Basic Life Sci *16*, 163-181.

Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., and Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science *270*, 96-99.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P., and Hogness, D. S. (1991). The Drosophila EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell *67*, 59-77.

Kucharova-Mahmood, S., Raska, I., Mechler, B. M., and Farkas, R. (2002). Temporal regulation of Drosophila salivary gland degeneration by the Broad-Complex transcription factors. J Struct Biol *140*, 67-78.

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

Lapie, P., Nasr, F., Lepesant, J. A., and Deutsch, J. (1993). Deletion scanning of the regulatory sequences of the Fbp1 gene of Drosophila melanogaster using P transposase-induced deficiencies. Genetics *135*, 801-816.

Laundrie, B., Peterson, J. S., Baum, J. S., Chang, J. C., Fileppo, D., Thompson, S. R., and McCall, K. (2003). Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. Genetics *165*, 1881-1888.

Laval, M., Pourrain, F., Deutsch, J., and Lepesant, J. A. (1993). In vivo functional characterization of an ecdysone response enhancer in the proximal upstream region of the Fbp1 gene of D. melanogaster. Mech Dev *44*, 123-138.

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

Lee, C. Y., and Baehrecke, E. H. (2001). Steroid regulation of autophagic programmed cell death during development. Development *128*, 1443-1455.

Lee, C. Y., Clough, E. A., Yellon, P., Teslovich, T. M., Stephan, D. A., and Baehrecke, E. H. (2003). Genome-wide analyses of steroid- and radiation-triggered programmed cell death in Drosophila. Curr Biol *13*, 350-357.

Lee, C. Y., Cooksey, B. A., and Baehrecke, E. H. (2002a). Steroid regulation of midgut cell death during Drosophila development. Dev Biol *250*, 101-111.

Lee, C. Y., Simon, C. R., Woodard, C. T., and Baehrecke, E. H. (2002b). Genetic mechanism for the stage- and tissue-specific regulation of steroid triggered programmed cell death in Drosophila. Dev Biol *252*, 138-148.

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.

Lemaitre, B., Reichhart, J. M., and Hoffmann, J. A. (1997). Drosophila host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc Natl Acad Sci U S A *94*, 14614-14619.

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 Rep *1*, 353-358.

Li, T., and Bender, M. (2000). A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in Drosophila. Development *127*, 2897-2905.

Li, X., Yang, Y., and Ashwell, J. D. (2002). TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2. Nature *416*, 345-347.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K.*, et al.* (2000). The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell *6*, 1389-1399.

Liu, Z., Sun, C., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C., and Fesik, S. W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. Nature *408*, 1004-1008.

Manfruelli, P., Reichhart, J. M., Steward, R., Hoffmann, J. A., and Lemaitre, B. (1999). A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. Embo J *18*, 3380-3391.

Maniatis, T., Sambrook, J., Fritsch, E.F. (1989). Molecular Cloning: A laboratory manual (2nd edition)).

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-1 beta-converting enzyme and related caspases. J Biol Chem *272*, 7223-7228.

Maroni, G., and Stamey, S. (1983). Use of blue food to select synchronous, late third instar larvae. Drosophila Inf Serv *59*, 142-143.

Martin, D. N., and Baehrecke, E. H. (2004). Caspases function in autophagic programmed cell death in Drosophila. Development *131*, 275-284.

Martin, S. J. (2002). Destabilizing influences in apoptosis: sowing the seeds of IAP destruction. Cell *109*, 793-796.

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

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.

Milan, M. (1998). Cell cycle control in the Drosophila wing. Bioessays *20*, 969-971.

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

Mugat, B., Brodu, V., Kejzlarova-Lepesant, J., Antoniewski, C., Bayer, C. A., Fristrom, J. W., and Lepesant, J. A. (2000). Dynamic expression of broad-complex isoforms mediates temporal control of an ecdysteroid target gene at the onset of Drosophila metamorphosis. Dev Biol *227*, 104-117.

Muro, I., Hay, B. A., and Clem, R. J. (2002). The Drosophila DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. J Biol Chem *277*, 49644-49650.

Neufeld, T. P., de la Cruz, A. F., Johnston, L. A., and Edgar, B. A. (1998). Coordination of growth and cell division in the Drosophila wing. Cell *93*, 1183-1193.

Nezis, I. P., Stravopodis, D. J., Papassideri, I., Robert-Nicoud, M., and Margaritis, L. H. (2000). Stage-specific apoptotic patterns during Drosophila oogenesis. Eur J Cell Biol *79*, 610-620.

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

Perez-Garijo, A., Martin, F. A., and Morata, G. (2004). Caspase inhibition during apoptosis causes abnormal signalling and developmental aberrations in Drosophila. Development *131*, 5591-5598.

Peterson, J. S., Barkett, M., and McCall, K. (2003). Stage-specific regulation of caspase activity in drosophila oogenesis. Dev Biol *260*, 113-123.

Puthalakath, H., and Strasser, A. (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ *9*, 505-512.

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.

Ress, C., Holtmann, M., Maas, U., Sofsky, J., and Dorn, A. (2000). 20-Hydroxyecdysone-induced differentiation and apoptosis in the Drosophila cell line, l(2)mbn. Tissue Cell *32*, 464-477.

Restifo, L. L., and White, K. (1991). Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of the central nervous system in Drosophila. Dev Biol *148*, 174-194.

Richards, G. (1976). Sequential gene activation by ecdysone in polytene chromosomes of Drosophila melanogaster. V. The late prepupal puffs. Dev Biol *54*, 264-275.

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.

Riddiford, L. M. (1993). Hormone receptors and the regulation of insect metamorphosis. Receptor *3*, 203-209.

Riedl, S. J., and Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol *5*, 897-907.

Robertson, C. W. (1936). The metamorphosis of *Drosophila melanogaster*, including an accurately timed account of the principle morphological changes. Journal of Morphology *59*, 351-399.

Robles, R., Tao, X. J., Trbovich, A. M., Maravel, D. V., Nahum, R., Perez, G. I., Tilly, K. I., and Tilly, J. L. (1999). Localization, regulation and possible consequences of apoptotic protease-activating factor-1 (Apaf-1) expression in granulosa cells of the mouse ovary. Endocrinology *140*, 2641-2644.

Rusconi, J. C., Hays, R., and Cagan, R. L. (2000). Programmed cell death and patterning in Drosophila. Cell Death Differ *7*, 1063-1070.

Ryoo, H. D., Gorenc, T., and Steller, H. (2004). Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev Cell *7*, 491-501.

Salvesen, G. S., and Abrams, J. M. (2004). Caspase activation - stepping on the gas or releasing the brakes? Lessons from humans and flies. Oncogene *23*, 2774-2784.

Schubiger, M., Wade, A. A., Carney, G. E., Truman, J. W., and Bender, M. (1998). Drosophila EcR-B ecdysone receptor isoforms are required for larval molting and for neuron remodeling during metamorphosis. Development *125*, 2053-2062.

Schwerk, C., and Schulze-Osthoff, K. (2003). Non-apoptotic functions of caspases in cellular proliferation and differentiation. Biochem Pharmacol *66*, 1453-1458.

Seshagiri, S., and Miller, L. K. (1997). Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED-3-induced apoptosis. Curr Biol *7*, 455-460.

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.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell *9*, 459-470.

Shiozaki, E. N., Chai, J., Rigotti, D. J., Riedl, S. J., Li, P., Srinivasula, S. M., Alnemri, E. S., Fairman, R., and Shi, Y. (2003). Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell *11*, 519-527.

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

Spector, M. S., Desnoyers, S., Hoeppner, D. J., and Hengartner, M. O. (1997). Interaction between the C. elegans cell-death regulators CED-9 and CED-4. Nature *385*, 653-656.

Spradling, A. C. (1993). Germline cysts: communes that work. Cell *72*, 649-651.

Stennicke, H. R., and Salvesen, G. S. (1999). Catalytic properties of the caspases. Cell Death Differ *6*, 1054-1059.

Sulston, J. E., and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Dev Biol *56*, 110-156.

Talbot, W. S., Swyryd, E. A., and Hogness, D. S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell *73*, 1323-1337.

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

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. Science *281*, 1312-1316.

Thummel, C. S. (1990). Puffs and gene regulation--molecular insights into the Drosophila ecdysone regulatory hierarchy. Bioessays *12*, 561-568.

Thummel, C. S., Boulet, A. M., and Lipshitz, H. D. (1988). Vectors for Drosophila P-element-mediated transformation and tissue culture transfection. Gene *74*, 445-456.

Truman, J. W., and Riddiford, L. M. (2002). Endocrine insights into the evolution of metamorphosis in insects. Annu Rev Entomol *47*, 467-500.

Truman, J. W., Talbot, W. S., Fahrbach, S. E., and Hogness, D. S. (1994). Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. Development *120*, 219-234.

van Gurp, M., Festjens, N., van Loo, G., Saelens, X., and Vandenabeele, P. (2003). Mitochondrial intermembrane proteins in cell death. Biochem Biophys Res Commun *304*, 487-497.

Vaux, D. L., and Flavell, R. A. (2000). Apoptosis genes and autoimmunity. Curr Opin Immunol *12*, 719-724.

Veis, D. J., Sorenson, C. M., Shutter, J. R., and Korsmeyer, S. J. (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell *75*, 229-240.

von Kalm, L., Crossgrove, K., Von Seggern, D., Guild, G. M., and Beckendorf, S. K. (1994). The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of Drosophila metamorphosis. Embo J *13*, 3505-3516.

Waldmeier, P. C., and Tatton, W. G. (2004). Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? Drug Discov Today *9*, 210-218.

Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c. Genes Dev *14*, 2060-2071.

Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science *292*, 727-730.

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.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D. A., Agapite, J., Steller, H., and Meier, P. (2002). The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol *4*, 445-450.

Wing, J., Zhou, L., Schwartz, L., and Nambu, J. (1999). Distinct cell killing properties of the Drosophila reaper, head involution defective, and grim genes. Cell Death Differ *6*, 212-213.

Wing, J. P., Schreader, B. A., Yokokura, T., Wang, Y., Andrews, P. S., Huseinovic, N., Dong, C. K., Ogdahl, J. L., Schwartz, L. M., White, K., and Nambu, J. R. (2002). Drosophila Morgue is an F box/ubiquitin conjugase domain protein important for grim-reaper mediated apoptosis. Nat Cell Biol *4*, 451-456.

Woodard, C. T., Baehrecke, E. H., and Thummel, C. S. (1994). A molecular mechanism for the stage specificity of the Drosophila prepupal genetic response to ecdysone. Cell *79*, 607-615.

Wu, D., Wallen, H. D., Inohara, N., and Nunez, G. (1997a). Interaction and regulation of the Caenorhabditis elegans death protease CED-3 by CED-4 and CED-9. J Biol Chem *272*, 21449-21454.

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

Wu, G., Chai, J., Suber, T. L., Wu, J. W., Du, C., Wang, X., and Shi, Y. (2000). Structural basis of IAP recognition by Smac/DIABLO. Nature *408*, 1008-1012.

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

Yan, N., Wu, J. W., Chai, J., Li, W., and Shi, Y. (2004). Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. Nat Struct Mol Biol *11*, 420-428.

Yao, T. P., Segraves, W. A., Oro, A. E., McKeown, M., and Evans, R. M. (1992). Drosophila ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell *71*, 63-72.

Yin, V. P., and Thummel, C. S. (2004). A balance between the diap1 death inhibitor and reaper and hid death inducers controls steroid-triggered cell death in Drosophila. Proc Natl Acad Sci U S A *101*, 8022-8027.

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

Yuan, J., and Yankner, B. A. (2000). Apoptosis in the nervous system. Nature *407*, 802-809.

Zhimulev, I., Belyaeva, E., Mazina, O. M., and Balasov, M. L. (1995). Structure and expression of the *BR-C* locus in *Drosophila melanogaster* (Diptera: Drosophilidae). European Journal of Entomology *92*, 263-270.

Zhou, B. B., Li, H., Yuan, J., and Kirschner, M. W. (1998). Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells. Proc Natl Acad Sci U S A *95*, 6785-6790.

Zimmermann, K. C., Ricci, J. E., Droin, N. M., and Green, D. R. (2002). The role of ARK in stress-induced apoptosis in Drosophila cells. J Cell Biol *156*, 1077-1087.

Zollman, S., Godt, D., Prive, G. G., Couderc, J. L., and Laski, F. A. (1994). The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. Proc Natl Acad Sci U S A *91*, 10717-10721.

Amendments

Chapter 3

- Figure 3.1C: The filled triangle indicating the position of the insertion in the *dronc* mutant complementation transgene should be closer to the *dronc* transcription start site (see chapter 3 bullet point 2 below).

- Insert p71, line 5: The *dronc* mutant complementation construct has an insertion following base 19 from the ATG. This causes a frame shift mutation disrupting translation of the entire *dronc* gene but preserves the promoter elements of the *CG6685* gene.

- Figure 3.3 A and B: Equal amounts of total protein were loaded. Molecular weight marker (not shown) corresponds to the indicated bands being DRONC protein.

- Figure 3.7 A: "$dronc^{d5}$" should be "$dronc^{Z}$". These animals were heterozygous.

- Figure 3.23 A: Line 3 of legend should read "mounted on a glass *haemocytometer* slide" instead of "mounted on a glass *microscope* slide".

Chapter 4

- Figures 4.3 B and 4.4 B, 5.2 B and 5.3B: Error bars represent the mean ± SD derived from three replicate analyses.

Chapter 5

- Figures 5.2 B, 5.3B and 5.8: Error bars represent the mean ± SD derived from three replicate analyses.