Regulation of the Drosophila Initiator Caspase Dronc through Ubiquitylation

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REGULATION OF THE DROSOPHILA INITIATOR CASPASE DRONC THROUGH UBIQUITYLATION

A Dissertation Presented

By

HATEM ELIF KAMBER KAYA

Submitted to the Faculty of the

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January 17, 2017

Cancer Biology Program
REGULATION OF THE DROSOPHILA INITIATOR CASPASE DRONC THROUGH UBIQUITYLATION

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Cancer Biology Program
January 17, 2017
This thesis is dedicated to my dear husband, my father, my mother and my brother.
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ABSTRACT

Apoptosis is a programmed cell death mechanism that is evolutionary conserved from worms to humans. Apoptosis is mediated by initiator and effector caspases. The initiator caspases carry long pro-domains for their interaction with scaffolding proteins to form a cell-death platform, which is essential for their activation. Activated initiator caspases then cleave effector caspases that execute cell death through cleaving downstream targets. In addition to their apoptotic function, caspases also participate in events where caspase activity is not required for cell killing, but for regulating other functions, so-called non-apoptotic functions of caspases. The *Drosophila* initiator caspase Dronc, the ortholog of mammalian caspase-2 and caspase-9 has a CARD domain that is essential for its interaction with the scaffolding protein Dark to form the apoptosome. Apoptosome formation is crucial for activation of Dronc. Activity of both initiator and effector caspases are further kept in control by the ubiquitin system to avoid inappropriate caspase activity. However, mechanistic details of how the ubiquitin system regulates activation of Dronc are not clear. Therefore, I investigated the ubiquitylation status of Dronc and its function in *Drosophila*. I found that Dronc is mono-ubiquitylated at Lys78 (K78) in its CARD domain, which blocks its interaction with Dark and formation of the apoptosome. Furthermore, I demonstrated that K78 mono-ubiquitylation plays an inhibitory role in Dronc’s non-apoptotic functions, which may not require its catalytic activity but may be important for the survival of the fly. This thesis study unveils the link between the ubiquitin system and caspases through a regulatory
mechanism where a single mono-ubiquitylation event could inhibit both apoptotic and non-apoptotic functions of a caspase.
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PREFACE

All work was performed at the University of Massachusetts Medical School in the laboratory of Andreas Bergmann.

Chapter II is published in:


Chapters I and III are unpublished including Figures 1.1 through 1.5 and Figure 3.1.
CHAPTER I

Introduction

Part I. Apoptosis

Cell death was first mentioned in the nineteenth century by anatomists who worked on metamorphosis of organisms such as tad poles and insects [1]. They used histological techniques to examine various tissues that undergo cell death [1]. However, the features of cell death were characterized in the twentieth century (early 1970s) by electron microscopy [2]. Australian pathologists John Kerr and his colleagues observed nuclear and cytoplasmic condensation of dying mammalian cells [2]. These changes cause cell shrinkage, DNA fragmentation and blebbing of the plasma membrane, which appears in a specific pattern [2]. Eventually, whole cells break up into ‘apoptotic bodies” that are being taken up by phagocytic cells to be ingested. They termed this type of cell death as “apoptosis” that originates from ancient Greek and means “falling off” [2].

Cell death is as important as cell survival is when it comes to homeostasis of organisms. During development of organisms, apoptosis occurs to shed extra or damaged cells and to drive morphogenesis for cellular rearrangements [3, 4]. In the presence of stress stimulus, however, apoptosis can aid dying cells to communicate with neighboring
cells by releasing mitogenic signals. This type of communication can induce tissue regeneration or tumor development [3, 4].

Apoptosis is a well-conserved cell death mechanism from worms to humans (Figure 1.3). Redundancy between apoptotic players increases in proportion to complexity of eukaryotes so as dissecting the mechanism of apoptosis becomes more challenging. Higher eukaryotes such as mammals have multiple pro- and anti-apoptotic regulators whereas lower eukaryotes such as *Drosophila melanogaster* have fewer players. The following sections will elaborate on mechanistic details of Caenorhabditis elegans, Drosophila and mammalian apoptotic machinery.

**A. Apoptotic Machinery in C. elegans**

First insights into the mechanism of apoptosis came from studies in the nematode Caenorhabditis elegans [5]. Examination of cell lineages of *C. elegans* by J. E. Sulston and his colleagues led to the discovery that 131 out of 1090 cells generated during *C. elegans* development undergo programmed cell death (apoptosis) [5, 6]. The first attempt to identify genes that are responsible for apoptosis was a genetic screen in *C. elegans*. This screen discovered *cell-death abnormal (ced)-1* and *ced-2* genes that are important for phagocytosis of apoptotic cells [7]. Further screens carried out by Robert Horvitz and his colleagues identified core proteins of the apoptotic pathway in *C. elegans*: *egg-laying defective (egl)-1, ced-9, ced-3, ced-4* [8-10] (Figure 1.3). Dying cells require EGL-1 (a pro-apoptotic protein with a BH3 motif), CED-3 (a caspase homolog) and CED-4 (homolog of Apaf1 and Dark) (Figure 1.3) [11]. In healthy cells, *ced-9* encodes a pro-
survival bcl-2 homolog, which protect cells from apoptosis by inhibiting ced-4 (Figure 1.3) [11]. In dying cells, EGL-1 blocks CED-9’s function to inhibit CED-4, which results in C. elegans apoptosome assembly and activation of CED-3 [11]. Induction of egl-1 expression by developmental cues or external signals determines which cells undergo cell death [11]. Strong loss of function mutations in egl-1, ced-3 or ced-4 blocks most apoptotic cell death; however, do not affect viability of C. elegans. Cells that lack the apoptotic machinery can differentiate into neural cells [11]. On the other hand, strong loss of function mutations in ced-9 cause embryonic lethality [11]. Studies to identify apoptotic players in C. elegans have helped the apoptosis field to advance with the discovery of the apoptotic pathways in Drosophila melanogaster and mammals, which are described in the next two sections.

**B. Apoptotic Machinery in Drosophila**

*Drosophila melanogaster*, also known as the fruit fly, has been used as a model organism more than one hundred years for genetic analyses. *Drosophila* has a short life cycle, taking 10 days from embryogenesis to eclosion at 25°C. The life cycle of *Drosophila* consists of 4 developmental stages; embryonic, larval, pupal and adult. (Figure 1.1) Development of an embryo into an adult requires apoptosis to remove unwanted cells and tissues to sculpt the final form of the organism. During embryogenesis, developmental apoptosis can be observed 7 hours after egg deposition (Stage 11) [12]. Apoptosis contributes to embryonic pattern formation (stages 12-14) that allows for segmentation of the embryo along the anterior-posterior axes by segment
polarity and homeotic genes [5]. In addition to segmentation, apoptosis is essential for Central Nervous System (CNS) maturation during embryogenesis. For example, midline glial cells die during stage 12-15 and reduce their numbers from 8-10 cells to 3 cells. One of the survival signaling pathways, Epidermal Growth Factor Receptor (EGFR) pathway is found to inhibit apoptosis to determine the final number of surviving cells in midline glia [5]. In addition, embryonic neuroblasts undergo apoptosis [13]. Completion of embryogenesis is followed by the larval phase, when epithelial imaginal discs proliferate in preparation to give rise to the final adult structures (Figure 1.1). The mature larva enters into metamorphosis, marking the start of pupal development where the pre-patterned tissues are then differentiated, reorganized, and sculpted leading to the final adult stage (Figure 1.1).

Cell death plays various roles in larva and pupa to remove larval structures that are not needed for the fully formed adult fly. Ecdysone receptor (EcR) signaling regulates apoptosis and metamorphosis during both larval and pupal stages [14, 15]. During mid-3rd instar, a low-dosage ecdysone pulse induces apoptosis through upregulation of pro-apoptotic genes [16]. A higher-dosage ecdysone pulse at the end of 3rd instar triggers metamorphosis through remodeling of tissues such as abdominal muscles via apoptosis [14]. A second ecdysone pulse at 12 hours after puparium formation initiates larval salivary gland degradation and larval fat body remodeling, which also requires apoptosis [14]. In addition, EcR signaling induces apoptosis for removal of a subset of neurons in the ventral nervous system, remodeling of the optic lobe and neuronal pruning throughout pupal development [14]. Independently of EcR
Figure 1.1. The Life Cycle of *Drosophila melanogaster*

The *Drosophila* life cycle consists of embryonic, larval, pupal and adult stages. At 25°C, *Drosophila* can develop from embryo to adulthood in 10 days. Embryos hatch 1 day after egg lay. Larval phase includes 1st, 2nd and 3rd instar stages which take about 4 days. Larva molts into pupa. Metamorphosis takes 4-5 days, which is followed by eclosion.
signaling, apoptosis is also observed to remodel pupal retinal cells where surplus interommatidial cells between each ommatidium of the Drosophila eye are eliminated through apoptosis [17-20]. Upstream regulation of apoptosis involves various signaling pathways that are controlled by developmental cues as exampled above. The remaining of this section will focus on the apoptotic cascade and its players in detail.

Drosophila entered the apoptosis field in the mid-90’s after a deficiency screen in embryos for identification of apoptotic genes uncovered the H99 locus (75C1,2) [21]. H99 mutant embryos were embryonic lethal and did not show any features of apoptosis in developing embryos. However, a high level of X-ray exposure of H99 mutant embryos displayed a few apoptotic cells [21]. This suggested that genes that are in the H99 locus are upstream regulators of apoptosis because effectors of apoptosis can still function in X-ray induced H99 mutant embryos. Reaper (rpr) was the first gene being cloned from the H99 locus [21]. After rpr, head involution defective (hid) was isolated from the H99 locus and shown to work in parallel to rpr for induction of apoptosis [22]. Interestingly, allelic combinations of hid mutant have adult male genitalia rotation defects and abnormal wing phenotypes [23]. These phenotypes were attributed to defects in cell death because both male genitalia rotation and wing development require caspase activation [24]. The last gene isolated from the H99 locus was grim which was shown to play a role in apoptosis in parallel to rpr and hid [25]. All three genes found in the H99 locus were able to induce apoptosis independently in both wild-type and H99 mutant embryos. Induction of apoptosis through activation of hid, rpr or grim can be suppressed by
baculovirus P35 (an effector caspase inhibitor) protein expression, suggesting that P35 and caspases act downstream of hid, rpr and grim [22] [25-27].

In an attempt to screen for cell death inhibitors through mutations that enhance rpr-induced cell death in the Drosophila eye, thread was identified as the Drosophila Inhibitor of Apoptosis 1 (DIAP1) (Figure 1.2) [28]. IAPs were first discovered in baculoviruses and can block apoptosis during viral infection [29]. Their characteristic features include at least one Baculovirus IAP Repeat (BIR) motif that is located in the N-terminus or central portion of IAPs and some have a C-terminally located RING domain [30]. A database search after identification of DIAP1 uncovered DIAP2 as the second IAP in Drosophila [28]. DIAP1 has two BIR domains; whereas DIAP2 has three BIR domains and both IAPs share a C-terminal RING domain [28]. They can suppress rpr and hid-induced or developmentally occurring cell death in the Drosophila eye [28]. In another screen, the Drosophila homolog of mammalian Bruce (dBruce) was characterized as the third IAP in flies. dBruce suppressed rpr- and grim-induced but not hid-induced cell death in the Drosophila eye [31, 32]. dBruce encodes an E2 conjugation enzyme with one BIR domain but does not function through ubiquitylation of RPR and GRIM [31, 32]. A fourth IAP in flies was identified with one BIR domain only and no RING domain, which was called Deterin [33] and is most similar to mammalian Survivin. Deterin was shown to suppress rpr-induced cell death in Drosophila S2 cells [33]. Interestingly, BIR-only or C-terminal-only Deterin could not inhibit ectopically induced apoptosis in cells [33]. In fact, C-terminal-only Deterin acted as a dominant negative construct by competing with wild-type Deterin [33].
The link between *Drosophila* IAPs, RPR, HID and GRIM came after two studies from the same group showed that DIAP1 and DIAP2 can interact with RPR, HID and GRIM physically through their BIR domains and this interaction could yield a depletion of RPR, HID and GRIM in SF-21 cells (Figure 1.2) [34, 35]. RPR, HID and GRIM share a conserved sequence of 14 amino acids (RHG motif) at their N-terminus [58, 15, 17]. In particular, first 37 amino acids of HID and GRIM were required and sufficient to induce apoptosis and bind to DIAP1 [58].

In a screen carried out to find modifiers of *rpr*- and *hid*-induced cell death in the *Drosophila* eye, both loss- and gain-of-function mutations in *thread* were recovered [36, 37]. To determine the importance of DIAP1 for cell viability, another study characterized embryonic *thread* homozygote mutants. *thread* mutant exhibited embryonic lethality associated with increased apoptosis [38]. Genetic and biochemical analyses of loss- and gain-of-function mutants showed that the BIR1 domain of DIAP1 binds to RPR and GRIM; whereas BIR2 interacts with HID (Figure 1.2) [36, 37]. Controversially, a structural study showed that the first 7 aa of HID, RPR and GRIM was sufficient for their interaction with DIAP1 BIR2, which was similar to the interaction between their mammalian counterparts, BIR3 of XIAP1 and Smac [39]. Two different groups characterized *sickle* (*skl*) as the new member of IAP antagonists [40, 41]. *skl* resides just outside of the *H99* locus and carries homology to N-terminal 4 residues in RPR, HID and GRIM. Alanine in the first position and proline on third position were highly conserved among RPR, HID, GRIM, Skl and their mammalian counterparts Smac/DIABLO and HtrA2/OMI [40-42]. Skl (1-15) appeared to interact with BIR2 of DIAP1; whereas, full
length Skl interacted with BIR1 of DIAP1 [40, 41]. Skl expression in *Drosophila* cells resulted in apoptosis that can be suppressed by P35 [40, 41].

The first link between RHG proteins and mitochondria came from a study identifying that the C-terminus of HID (393-409) carries a Mitochondrial Targeting Sequence (MTS) and is necessary for mitochondrial localization in mammalian and SF9 cells [43]. Deletion of the MTS (HID (393-409)) resulted in abrogation of mitochondrial localization in mammalian cells; however, it did not affect its function [43]. In contrast, *hid*\textsuperscript{A206} and *hid*\textsuperscript{A329} truncation alleles were characterized as loss-of-function mutants in *Drosophila* [43]. Among the sequences of GRIM to form an alpha-helix structure Grim Helix 3 (GH3) with 15 aa was identified. The GH3 had homology to same regions in RPR and Skl [44]. Both the N-terminus of GRIM and its GH3 region were necessary for apoptosis-inducing ability of GRIM in *Drosophila* independent from each other [44]. Interestingly, GH3 was essential for GRIM to associate with mitochondria and also co-localize with cytochrome c [44]. In addition to GRIM, the GH3 region of RPR was characterized to be necessary and sufficient to localize to the mitochondria in S2 cells [45]. Further examination discovered that the GH3 region interacts with the outer membrane of mitochondria and this interaction enhances *rpr*-induced apoptosis [46]. The GH3 region mutant *rpr* (*rpr*\textsuperscript{AGH3}) could retain its interaction with DIAP1 and its ability to suppress DIAP1-mediated caspase inhibition in yeast cells [45]. However, *rpr*\textsuperscript{AGH3} mutant cannot induce DIAP1 degradation in S2 cells, which can be restored by targeting *rpr*\textsuperscript{AGH3} mutant to the mitochondria [45]. A study to examine the relationship between RHG proteins and their ability to interact with each other and the mitochondria discovered that
Figure 1.2. IAP Antagonists bind to BIR1 and BIR2 domains of DIAP1 to inhibit its interaction with caspases.

Upon apoptotic stimuli, RPR, GRIM and HID oligomerize and attach to the mitochondria for a better cell killing activity. RPR and GRIM bind to both BIR1 and BIR2 domains of DIAP1; whereas, HID binds to BIR2 of DIAP1, which causes DIAP1 auto-degradation. These interactions block binding of Drice and Dcp-1 to BIR1 of DIAP1 and Dronec to BIR2 of DIAP1. On the other hand, in living cells, DIAP1 could ubiquitylate caspases and IAP antagonists for their inhibition.
RPR associates with itself and GRIM and HID but not Skl through its GH3 region [47]. The MTS of HID was required for recruitment of RPR and DIAP1 to the mitochondria, where RPR induces DIAP1 degradation [47]. This study suggested that forming a multimeric RHG complex at the mitochondrial membrane provides stability for these proteins and enhance degradation of DIAP1 to induce cell death cooperatively and efficiently (Figure 1.2) [47].

In addition to Skl, another IAP antagonist in *Drosophila* was identified in the same year through tandem affinity purification followed by mass-spectrometry; a thioredoxin peroxidase Jafrac2 [48]. Jafrac2 interacts with the BIR2 domain of DIAP1 and carries the same conserved sequences with other IAP antagonists at its N-terminus [48]. Interestingly, Jafrac2 was found to localize and release from the Endoplasmic Reticulum (ER) to the cytosol upon apoptosis induction [48]. Interestingly, another study showed that DIAP1 when targeted to the ER, away from the mitochondria can be degraded by RPR<sup>wt</sup> and RPR<sup>ΔGH3</sup> mutant, suggesting that intracellular membranes could act as scaffolds for DIAP1 degradation [46]. Jafrac2 also can induce apoptosis in S2 cells and in the *Drosophila* eye by competing with Dronc for binding to the BIR2 of DIAP1 [48]. Further examination of interactions between IAP antagonists and DIAP1 in S2 cells identified that RPR and GRIM bind to BIR1 and BIR2 of DIAP1 with equally efficiency; whereas HID, Skl and Jafrac2 preferentially bind to BIR2 of DIAP1 (Figure 1.2) [49].

The apoptotic pathway requires the assembly of a large protein complex critical for responding to the developmental or stress-induced cues that trigger activation of
caspases (Cysteine-Aspartic proteases) (Figure 1.3) [50]. All caspases are initially produced as inactive enzymes, called zymogens. These zymogens contain a large and a small subunit [50]. The region required for catalytic activity is located within the large subunit [50]. There are two types of caspases; initiator and effector caspases (Figures 1.3 and 1.4). The difference between initiator and effector caspases is that initiator caspases have a long pro-domain for interacting with scaffolding proteins [50]. This interaction induces their dimerization that is followed by auto-cleavage between their large and small subunits [50]. Activated and mature initiator caspases cleave effector caspases for their activation to initiate apoptosis (Figures 1.3 and 1.4). Once activated, effector caspases cleave downstream targets to dismantle the cells.

Because rpr, hid and grim-induced cell death can be suppressed by P35, efforts to identify Drosophila caspases through degenerate PCR-based methods revealed two Drosophila effector caspases: Drosophila Interleukin-1β-Converting Enzyme (drICE) and Drosophila Caspase-1 (DCP-1) [51-54]. These caspases share 57% amino acid identity with each other and are homologs of mammalian caspase-3 and caspase-7 [52-54]. In addition, they both have a catalytic cysteine with conserved QACQG sequence and a short pro-domain [52-54]. After discovery of Drosophila caspases, the next question was whether or not they could be inhibited by IAPs. In this direction, studies in SF-21 cells showed that DIAP1 but not DIAP2 can block apoptosis induced by drICE [55]. Further analysis suggested that drICE can be processed by HID activation and the processed version but not the pro-enzyme can physically interact with DIAP1 [55].
Apoptosis is an evolutionary conserved mechanism from worms to mammals. Apoptotic stimuli induce pro-apoptotic proteins to inhibit IAPs. This inhibition promotes activation of the initiator caspases through formation of the apoptosome. Activated initiator caspases further activate effector caspases through cleavage to execute apoptosis.
Another study that was carried out in yeast with DIAP1 and DCP-1 showed that DIAP1 can block activity of DCP-1 [56].

The third caspase identified in Drosophila was dredd [57]. Besides carrying features of the caspase family, dredd has a long pro-domain which was distinct from drICE and dcp-1 and similar to mammalian caspase-8 [57]. Another difference between dredd and other Drosophila caspases was the position of the catalytic cysteine, which is encompassed by QACQE sequence where Glycine is replaced by Glutamine. Heterozygosity at dredd can suppress rpr- and grim-induced apoptosis in the eye [57]. Expression of rpr, hid or grim can induce DREDD processing in Drosophila cells and this processing was insensitive to P35 expression [57]. This was the first clue of existence of Drosophila initiator caspases. The second initiator and fourth caspase characterized in Drosophila was Dronc through a database search [58]. Dronc is the only Drosophila caspase identified to carry a Caspase Recruitment Domain (CARD) and has a 46% similarity to the mammalian caspase-2 [58]. Dronc has a catalytic cysteine, encompassed with PFCRG which is distinct from sequences found in other caspases [58]. The same study also reported that dronc expression in NIH 3T3 cells induced apoptosis that could be suppressed by P35 [58]. In vitro experiments showed that Dronc can undergo processing followed by activation [58]. In addition, dronc was the first caspase in Drosophila that is found to be regulated by ecdysone signaling [58].

First null mutants of Dronc (dronc<sup>d5</sup> and dronc<sup>51</sup>) were generated by P-element insertions. These mutants were pupal lethal [59, 60]. dronc nulls showed suppression of
rpr-, hid-, grim-induced apoptosis in the Drosophila eye and did not exhibit developmental cell death in the Drosophila embryo, retina and salivary glands [59, 60]. dronc null mutants exhibited hid mutant-like phenotypes such as defects in head involution in embryos, extra scutellar bristles and darkening of the wings [59, 60]. In addition, these studies indicated that maternal contribution of dronc mRNA is essential for dronc mutants to survive until late pupal stages [59, 60]. Dronc was also shown to be required for radiation and stress-induced apoptosis [59, 60]. Further null mutants of dronc (droncI24 and droncI29) recovered from an EMS screen also suppressed rpr- and hid-induced cell death in the Drosophila eye [61]. Developmental apoptosis in the Drosophila embryos, wings and retina could not function because of dronc null mutants [61]. This study also clarified the opaque wing phenotype that was observed previously [61]. A few escapers of dronc null mutants displayed less transparent, blemished and down-curved wings due to lack of apoptosis [61]. dronc germ line clones were embryonic lethal, also suggesting the importance of maternal contribution of dronc transcript [61].

The fifth Drosophila caspase to be characterized was Drosophila Executioner Caspase related to Apopain/Yama (DECAY) [62]. DECAY was found to be similar to mammalian caspase-3 and caspase-7 in homology and in substrate specificity [62]. The sequence encompassing its catalytic cysteine is QACRG which is shared by caspase-3 and 7 [62]. Expression of decay in 293T and NIH 3T3 cells leads to apoptotic morphology, confirming its ability to induce apoptosis [62].
Another database search for caspases yielded identification of the sixth and the seventh *Drosophila* caspases; Ser/Thr Rich Caspase (STRICA) and Death-Associated Molecule related to Mch-2 (DAMM) [63, 64]. DAMM has a high similarity to mammalian caspase-6 and 3; whereas, STRICA is more similar to caspase-8 with a long pro-domain lacking any CARD or Death Effector Domain (DED) domain [63, 64]. STRICA could interact with DIAP1 and DIAP2 *in vitro*; whereas DAMM could not interact with either of the IAPs [63, 64]. Additionally, both caspases did not form a complex with P35 [63, 64]. STRICA could induce apoptosis when expressed in *Drosophila* cells [64]. Similarly, expression of DAMM induced apoptosis in NIH 3T3 cells. In addition, expression of DAMM in the *Drosophila* eye caused a rough eye phenotype [63]. *rpr*-induced cell death could be suppressed by catalytically inactive DAMM in the *Drosophila* eye [63].

The idea of inducing cell death through RPR, HID and GRIM by disrupting interactions between caspases and DIAP1 came from a study conducted in yeast [38]. This study showed that co-expression of DCP-1 and DIAP1 in yeast cells did not result in death; however, co-expression of either RPR, HID or GRIM along with DCP-1 and DIAP1 killed yeast cells, suggesting that RPR inhibits DIAP1’s ability to block DCP-1 [38]. Co-expression of DIAP1, DCP-1 and the N-terminal 37 residues of HID (HID(1-37)) was also sufficient to kill yeast cells [38]. Another study also discovered that the BIR1 domain of DIAP1 interacts with processed drICE and DCP-1 [49]. Interestingly, both pro-Dronc and either activated drICE or DCP-1 can bind to BIR2 and BIR1 of DIAP1 simultaneously (Figure 1.2) [49].
A strong hypomorph of *drice*, *drice*\(^{17}\) and a null allele of *dcp-1*, *dcp^{Δ1}\) were characterized [65, 66]. Homozygous *drice*\(^{17}\) and *dcp-1^{Δ1}\) flies exhibited opaque wing phenotype, in agreement with *hid* and *dronc* mutants [65, 66]. *drice*\(^{17}\) and *drice^{Δ1}\) suppressed developmental apoptosis and partially protected against radiation-induced cell death [65, 66]. Homozygous *drice*\(^{17}\) and *drc^{Δ1}\) cannot completely abolish cell death in *Drosophila* embryos [65, 66]. However, apoptosis was reduced in double homozygous *dcp-1^{Prev1}\) (a null allele) [67] and *drice*\(^{17}\) mutant embryos [65]. Heterozygosity at *dcp-1* caused *drice^{Δ1}\) flies to die in late larval or early pupal stages [66]. This suggests that DCP-1 and drICE can be functionally redundant in *Drosophila* [65, 66]. Further examination of roles of drICE and DCP-1 in apoptosis uncovered that both caspases were activated upon irradiation-induced apoptosis in *Drosophila* larval wing [68]. Interestingly, DCP-1 cannot trigger radiation-induced apoptosis in the absence of drICE; whereas drICE can exert apoptosis in the absence of DCP-1 in the *Drosophila* wing [68]. The reason for this observation was that drICE can cleave some of caspase substrates more efficiently than DCP-1 *in vivo*; however, DCP-1 can cleave drICE and make it a more potent apoptotic inducer [68]. This suggest that intrinsic abilities of these caspases and their levels in cells are determinants of the cell death [68].

More mechanistic details of caspase activation came from a yeast two-hybrid assay [69]. This study revealed that pro-drICE can dimerize without its short pro-domain region even in its inactive form [69]. In addition, Dronc can interact with and cleave drICE [69]. This study also indicated that BIR2 domain of DIAP1 binds to pro-Dronc [69]. Lastly, reducing gene dosage of *dronc* can suppress the *rpr*- or *hid*-induced
Drosophila eye phenotype [69]. Each caspase showed different substrate cleavage specificity towards lamin, confirming the difference in sequences of their active sites [69]. Another study about Dronc’s cleavage specificity showed that Dronc can cleave itself after glutamate at TQTE$^{352}$; however, it cleaves drICE after aspartate at TQTD$^{230}$ [70]. Interestingly, DCP-1 can also cleave drICE at the same position [70]. This report also suggested that DCP-1 and drICE can cleave Dronc at its pro-domain and drICE can also remove its own pro-domain after cleaved by Dronc [70]. Both studies demonstrated that catalytically inactive Dronc suppressed rpr-, hid-, grim-induced cell death in the Drosophila eye, suggesting a dominant negative role for the catalytically inactive Dronc [69, 70]. Interestingly, Dronc could not cleave the caspase inhibitor P35 unlike drICE and DCP-1 [70].

Another main player of Drosophila apoptosis, Drosophila homolog of Apaf-1 was identified by three different groups under three different names: Dark, Dapaf-1 and HAC-1 (Figure 1.4) [71-73]. Drosophila Apaf-1-related killer (Dark) was identified by database searches and found to have an N-terminal CARD domain for interaction with caspases [71]. In the same study Dark was suggested to interact with Dredd and cytochrome c [71]. The authors also reported that a hypomorphic allele of dark (dark$^{CD4}$) has developmental abnormalities, but can develop to adulthood [71]. In addition, this mutant had less apoptosis in embryos and suppressed RPR, HID and GRIM induced cell death in the eye [71]. Drosophila Apaf-1 (Dapaf-1) was identified by degenerate PCR and found to have two isoforms; a short one (dapaf-1S with 531 aa) and a long one (dapaf-1L with 1440 aa - the major form in Drosophila) [72]. Both isoforms can activate
caspases in *Drosophila* S2 cells [72]. Immunoprecipitation experiments in S2 cells also indicated that drICE can bind to Dapaf-1S and Dronc can interact with Dapaf-1L, suggesting different specificities for these isoforms and first evidence of a Dark-Dronc complex (Figure 1.4) [72]. Loss-of-function mutant of *dapaf-1* (*dpf*\(^{K1}\)) exhibited reduced apoptosis in embryos and suppressed RPR-induced cell death in the eye, in parallel to the previous study [72]. This mutant was 25% semilethal during pupal stage [72]. Further examination of *dpf*\(^{K1}\) displayed extra cells that did not undergo apoptosis in neural cells during development [72]. Homolog of Apaf-1 and CED-4 (HAC-1) was also identified through a database search [73]. The findings of the other two groups were also confirmed in this study [73]. In addition, the authors made a *hac-1* null mutant (*hac-1*\(^{k11502}\)) which was embryonic lethal [73]. Recovery of additional null *dark* mutants from *hid*-induced cell death in the *Drosophila* eye indicated that Dark is essential for *Drosophila* development because homozygous null *dark* mutants were pupal lethal [74]. Just like *dronc*, maternal contribution of *dark* mRNA also lasts until late larval stages, allowing flies to develop to pupal stages [74]. Homozygosity at *dark* can inhibit most, but not all cell death in *Drosophila* embryo [74]. Dark was also shown to be required for irradiation-induced apoptosis [75]. Another study in *Drosophila* S2 cells suggested that downregulation of DIAP1 can be suppressed by downregulation of Dronc and/or Dark, indicating that in addition to Dronc, Dark is epistatic to DIAP1 [76]. This study also displayed that expression of catalytically inactive Dronc could enhance cell death induced by DIAP1 downregulation, suggesting an effector caspase independent pathway [76]. In confirmation with the previous study, DIAP1 was also shown to increase *hid* and
grim-induced apoptosis in the Drosophila eye that could be suppressed by darkCD4 mutant [77].

Because DIAP1 bears a RING domain, its importance in cell viability and regulation of caspases was investigated. diap1RING mutant flies failed to suppress rpr-induced cell death, alleviated hid-induced cell death and enhanced dronc-induced cell death in the Drosophila eye, suggesting a requirement of RING domain for DIAP1’s function [78]. Moreover, diap1RING mutant can still preserve its interaction with RPR, HID and Dronc [78]. The same study also exhibited the first evidence for E3 ligase activity of DIAP1 towards itself and Dronc [78]. This study also made the point that both interaction between DIAP1 and caspases and E3 ligase activity of DIAP1 are necessary and sufficient for inhibition of caspases [78]. Because DIAP1 can ubiquitylate Dronc in vitro, the next question was what does this ubiquitylation do to Dronc protein. Upon downregulation of DIAP1 or UV treatment, proteolytically processed large and small subunits of Dronc accumulated in S2 cells [79]. Treatment of S2 cells with proteasome inhibitor MG132 also caused accumulation of large processed version of Dronc [79]. However, downregulation of Dark resulted in accumulation of full-length Dronc [79]. Overall, this study suggested that Dronc is continuously processed in S2 cells, which is dependent on Dark and Dronc could be degraded by DIAP1 [79]. Another study about Dronc’s fate upon RPR, HID, GRIM-mediated apoptosis induction indicated that Dronc has a 12 residue IAP Binding Motif (IBM) between its CARD domain and large subunit that interacts with BIR2 of DIAP1 [80]. This motif is similar to the RHG domain [80]. Mutation in this domain of Dronc generated a more potent apoptotic inducer in the
Drosophila eye, which cannot be suppressed by DIAP1 [80]. Interestingly, Dronc and RHG proteins bind to the same surface groove in DIAP1, suggesting a competition between IAP antagonists and caspases [80]. In fact, in vitro ubiquitylation assay showed that addition of a10-residue peptides from the N-termini of Hid, RPR, GRIM or Sickle eliminated DIAP1-mediated Dronc ubiquitylation [80]. The authors also suggested that two caspase cleavage sites preceding the IBM of Dronc could regulate its function upon apoptosis induction by removing the IBM and eliminating its binding to DIAP1 [80]. One of these caspase cleavage sites at D135 of Dronc was later found to be mediated by Drice and had no effect on Dronc’s activity [81]. However, auto-cleavage of Dronc at D352 between large and small subunits was shown to be essential for Dronc’s activity [81]. This cleavage was dependent on Dark [81]. Another study confirmed the competition between RHG proteins and caspases to DIAP1. In this study, the authors showed that RPR and GRIM and surprisingly Jafrac2 but not HID abrogated binding of drICE or DCP-1 to BIR1 and BIR2 of DIAP1 [49].

As DIAP1 could mediate auto-ubiquitylation, a study asked the question whether or not this ubiquitylation depends on RHG proteins. This study conducted in vivo found that induction of RPR led to reduction of DIAP1 protein level which was dependent on the RING domain of DIAP1. In search for mediators of RPR-induced DIAP1 degradation, the authors identified UBCD1, E2-ubiquiting conjugating enzyme [82]. Loss of one functional copy of ubcD1 was able to suppress rpr-, hid- and diap1RING-induced cell death in the Drosophila eye [82]. This genetic analysis was supported by biochemical assays that showed interaction between RPR or GRIM, UBCD1 and DIAP1 [82, 83].
This interaction promoted auto-ubiquitylation of DIAP1 [82]. Further investigation of DIAP1 degradation upon induction of apoptosis through expression of \textit{rpr}, \textit{hid} or \textit{grim} identified that all three apoptotic inducers could reduce DIAP1 protein level in \textit{Drosophila} embryos [84]. In particular, HID could induce DIAP1 auto-degradation; whereas RPR and GRIM could exhibit inhibition of general protein translation [84, 85]. This report also suggested that DIAP1 is a short-lived protein with 40 min half-life and one of its targets, Dronc, has a longer half-life about 3 hours in S2 cells [84]. As a secondary layer of DIAP1 regulation, DIAP1 was characterized as the first protein to undergo degradation through N-end rule pathway in \textit{Drosophila} [86]. DIAP1 has a caspase cleavage site DQVD\textsuperscript{20}NN at its N-terminus, which is a target of DCP-1/drICE [86]. Cleaved DIAP1 could be stabilized by inhibition of the proteasome and downregulation of the N-end rule pathway in S2 cells [86]. Degradation of DIAP1 through N-end rule pathways is not dependent on its RING domain but its Asparagine-bearing N-degron [86]. Interestingly, this cleavage was necessary for DIAP1 to suppress \textit{rpr}-induced cell death [86]. Repression of the N-end rule pathway in the \textit{Drosophila} eye presumably stabilized DIAP1 and enhanced \textit{rpr}-induced cell death and alleviated \textit{hid}-induced cell death in the eye, similar to observations with \textit{diap}\textsuperscript{\textast{}RING} mutant [78, 86].

Other than ubiquitylating itself and Dronc, DIAP1 was also shown to ubiquitylate RPR, HID and GRIM in cells [87]. Further examination of this phenomenon uncovered that RPR is degraded by DIAP1, which is dependent on RHG domain of RPR (RPR (1-15)) and RING domain of DIAP1 [87]. Inhibition of the proteasome or mutating lysine residues of RPR to arginine to block ubiquitylation resulted in a stable RPR protein that
could induce apoptosis more potently [87]. In addition, another study reported that RPR, HID and GRIM could not induce apoptosis in S2 cells if their IBM domains are mutated [49].

The first detailed mechanism of effector caspase ubiquitylation by DIAP1 came from a study showed that DIAP1 poly-ubiquitylates and inhibits drICE and DCP-1 in a non-degradative manner [88]. In particular, cleavage of 20 aa of DIAP1 (due to the N-end rule pathway) provided a better interaction with drICE and DCP-1 and recruited UBR1 to promote ubiquitylation of effector caspases together with RING domain of DIAP1 [88]. Interestingly, DIAP1-mediated drICE ubiquitylation does not lead to proteasomal degradation but hinders the substrate interaction of drICE allostERICALLY [88]. Non-ubiquitable drICE could bind to DIAP1 but was still active [88]. In a model the authors suggested that DIAP1 resides in a ‘closed’ form and activation of effector caspases leads to cleavage of DIAP1 to generate an ‘open’ form which could in turn inhibit effector caspases [88]. In this model, the half-life of DIAP1 is rate-limiting for negative regulation of caspases [88]. In addition to effector caspases, Dronc was shown not to undergo proteasomal degradation upon increasing or decreasing the protein level of DIAP1 in Drosophila larval eye discs [89]. Extracts from DIAP1 RING mutant embryos also indicated that Dronc protein levels do not change compared to wild type embryonic extracts [89]. Furthermore, Dronc is processed in the presence of diap1\textsuperscript{ARING} mutant, suggesting that DIAP1 controls Dronc’s activity through a non-degradative manner in vivo [89].
Figure 1.4. The activation mechanism of Dronc

Upon apoptosis induction, Dronc is recruited to the Dark apoptosome through homotypic CARD interactions. Apoptosome is formed in 1:1 protein stoichiometry. Dronc is auto-cleaved in the apoptosome, which forms a homodimer consisting of its large and small subunits. The caspase domain is released from the apoptosome and cleaves effector caspases for their activation.
Involvement of mitochondria in *Drosophila* apoptosis has been debated over the years. Although early studies suggested that cytochrome c binds to Dark and induces cell death, other studies showed the controversial results such as cytochrome c is not released upon UV induction and loss of cytochrome c cannot suppress UV-induced and *rpr* or *grim*-induced apoptosis [90, 91]. Cytochrome c from mitochondria was not released and remained attached to mitochondria when Dronc and drICE were activated and led to death of *Drosophila* BG2 cells [92]. Interestingly, Dronc and active drICE partially located to mitochondria in serum-deprived BG2 cells. The same study also provided the first evidence of *Drosophila* apoptosome formation through gel filtration assays of BG2 cell extracts. In contrast, cytochrome c addition accelerated the formation of the apoptosome [92].

Structural insights of the *Drosophila* apoptosome came from two studies (Figure 1.4) [93, 94]. First study uncovered that Dark apoptosome consists of two rings that contain 8 Dark molecules [103]. Interestingly, cytochrome c did not interact with Dark in the apoptosome [103]. Further insights into the structure of the *Drosophila* apoptosome revealed that the CARD domains of Dronc and Dark form a crown-like structure at the central hub of the *Drosophila* apoptosome (Figure 1.4). The single-ring apoptosome was found to be sufficient to activate Dronc and represented the physiological state of the apoptosome. However, a more recent study recovered the two-ring apoptosome structure with 16 Dark and 16 Dronc molecules at a higher resolution [95]. Interestingly, formation of the apoptosome was triggered by addition of Dronc with or without dATP. The CARD domain of Dronc was essential for recruitment into the apoptosome. Once the
apoptosome was formed, Dronc was activated and caspase domain of Dronc that bears large and small subunits is released from the apoptosome (Figure 1.4). Atomic interactions between the CARD domains of Dronc and Dark showed that each CARD of Dronc interacts with neighboring CARD of Dronc through van der Waals contacts and with two different CARDs of Dark through Hydrogen bonds.

Supportive of above-mentioned studies, mutants affecting the WD40 domains of Dark exhibited a loss-of-function phenotype, suggesting that WD40 domain of Dark may function in a pro-apoptotic manner [74]. Interestingly, in the recent structural report the WD1 repeat of Dark was shown to interact with the CARD of Dronc, which is essential for formation of the multimeric apoptosome complex [95]. This finding could link the two studies together, where a pro-apoptotic WD40 repeat functions by interacting with the CARD of Dronc in the apoptosome possibly further stabilizing it. The second study showed that auto-cleaved Dronc undergoes homo-dimerization after cleavage at D352 to enhance its activity; whereas Dronc zymogen exists as a monomer [104]. Dronc also undergoes another cleavage to release its CARD domain at D135, which suggests that activated Dronc does not remain in the apoptosome unlike mammalian caspase-9 [104].

Two studies on activation mechanism of Dronc highlighted that auto-cleavage of Dronc at D352 was not essential for its activation and for inducing cell death in Drosophila BG2 or SL2 cells [96, 97]. Dronc could cleave both after aspartate (drICE) and glutamate (itself); however, prefers Asp on its substrates [96, 97]. Interestingly, these studies also suggested that auto-cleavage of Dronc could be a stabilizing factor for Dronc dimerization, which is essential for its activity after recruitment into the Dark
apoaptosome [96, 97]. This phenomenon is similar to mammalian caspase-2 and caspase-9 activation, as they also do not undergo an initial auto-cleavage for their activation [96, 97]. Interestingly, Dronc is activated in the apoptosome only after addition of cytosolic fractions, suggesting a requirement of a cytosolic factor other than cytochrome c in Dronc’s activation [96].

Dronc’s activity was shown to be regulated by metabolic input in Drosophila S2 cells [98]. This interesting study uncovered an inhibitory phosphorylation site for Dronc at S130, which is responsive to increased NADPH levels and mediated by CamKII kinase [98]. S130 phosphorylation of Dronc can inhibit its interaction with Dark; however, it does not affect its catalytic activity [98]. Suppression of Dronc activity through S130 phosphorylation was also observed in specific neural tissues of Drosophila [98].

Regulation of the Drosophila apoptosome activity was shown to be regulated by ‘feedback inhibition’ mechanism, in which excess levels of Dronc or Dark are degraded through a mechanism requiring catalytic activity of Dronc, the C-terminal of Dark and DIAP1 [99]. Dark carries a Dronc cleavage site at 1292 [100]. A non-cleavable Dark mutant could not be degraded through feedback inhibition mechanism [99]. This mechanism could ensure cell viability in times of accidental amplification of apoptosome activity [99].

There are two isoforms of cytochrome c in Drosophila, dc3/cyt c-p (the major form) and dc4/cyt c-d [101]. Examination of mutants of these isoforms revealed that neither of them are required for caspase activation in S2 cells or in flies, suggesting
further that cytochrome c is not required for Dark-mediated Dronc activation [102]. Interestingly, the dc4/cyt c-d isoform was found to be required for caspase activation during spermatid individualization [103] (discussed in detail in Chapter I, Part ID2 and Part IIIB). Further examination of DC3/Cyt c-p revealed that this isoform is required for mitochondrial respiration [104]. In addition to its role in spermatid individualization, DC4/Cyt c-d was also shown to be required for extra interommatidial cell death in developing *Drosophila* eye [105].

Homologs of Bcl-2 family proteins were identified in *Drosophila*. For example, Debcl was reported to be a pro-apoptotic Bcl-2 family member and has a correlating expression level with developmental cell death [106]. *debc* is epistatic to apoptosome activation [106]. *debc*-induced apoptosis cannot be suppressed by *H99* deficiency, but can be rescued by losing one functional copy of DIAP1 [106]. *buffy*, another Bcl-2 family member was conversely shown to be anti-apoptotic by blocking developmental and radiation-induced cell death [107]. *buffy* interacted with *debc* both genetically and physically to block its function [107]. On the other hand, *rpr*, *hid* or *grim*-induced cell death could be suppressed by *buffy* expression in the *Drosophila* eye [107]. Cell death-induced by *thread* heterozygosity was also suppressed with *buffy* expression in the *Drosophila* embryo [107]. Debcl localized to mitochondria but did not result in cytochrome c release [92]; whereas, Buffy resided in ER [108]. However, both proteins are shown to act conversely in a context dependent manner [109]. Because, homozygous *debc* or *buffy* null mutant flies develop normally, these two Bcl-2 homologs are not essential for viability and developmental apoptosis but may be required in specific tissues
and in stress-induced conditions [110]. In addition to Bcl2 family homologs, a
*Drosophila* homolog of Omi/HtrA2 (dOmi) was characterized [111]. dOmi can bind to
BIR2 of DIAP1 through two IBM domains that are exposed after dOMI is recruited to the
mitochondria and undergoes proteolytic cleavage [111]. This interaction can displace
Dronc and degrade it proteolytically [111]. dOmi was also shown to induce apoptosis
when expressed in the *Drosophila* eye, which can be suppressed by DIAP1 expression
[111, 112]. dOmi was also a target of DIAP1 ubiquitylation [112].

In another study, mitochondrial dynamics were examined upon *rpr*- or *hid-
expression in S2 cells [113]. HID and RPR could localize to mitochondria to induce
mitochondrial permeabilization and changes in mitochondrial ultrastructure, independent
of their function on DIAP1 [113]. Inhibition of mitochondrial disruption blocked
apoptosis [113]. The role of mitochondria in *Drosophila* apoptosis seems to be important;
however, it is not similar to mammalian apoptosis. Often times, mitochondrial elements
contribute to *Drosophila* apoptosis in a context- and tissue-dependent manner.

Conservation of the apoptotic pathway between *Drosophila* and the mammals, the
lack of redundancy among the apoptotic factors and the short life cycle have made
*Drosophila* an excellent model system to dissect apoptotic machinery over the years as
discussed in detail above. Mammalian apoptosis also has distinct features and has been
studied extensively to understand its mechanism due to its relevance to human diseases.
The following section will examine mammalian apoptotic cascade in detail.

**B. Apoptotic Machinery in Mammals**
There are two apoptotic pathways in vertebrates: extrinsic and intrinsic apoptosis [114]. While the extrinsic pathway relies on receptor-mediated signaling through a subset of Tumor Necrosis Factor Receptors (TNFR) in response to inflammation or infection, the intrinsic pathway is regulated by a mitochondria response to cytotoxic stimuli such as DNA damage or ER stress [114, 115]. Both of these pathways utilize the same effector caspases to execute apoptosis.

A subset of TNFRs that play a role in extrinsic apoptosis are called Death Receptors (DR) [116]. DR have an extracellular Cysteine-rich domain and an intracellular death domain of ~80 amino acids. DR transmit apoptotic signals through death ligands [115, 117]. Examples of well-characterized death receptor/ligand pairs are TNFR1/TNFα, FasR/FasL and TRAIL/TRAIL-R [118]. To transmit death signals, ligands bind to and trimerize their cognate receptors [114]. In the case of FasR/FasL and TRAIL/TRAIL-R, this induces recruitment of adaptor protein Fas-Associated protein with Death Domain (FADD) and initiator caspase-8 through interactions between their death domains to form the Death-Inducing Signaling Complex (DISC) [114, 117]. In this complex, caspase-8 dimerizes for its activation and then undergoes auto-cleavage between its large and small subunits for stabilization. Another cleavage occurs between the pro-domain and the large subunit, which releases caspase-8 from DISC. Activated caspase-8 can cleave and activate caspase-3 to execute apoptosis. In the case of TNFR1/TNFα, this ligand/receptor complex induces recruitment of TNFR1-Associated via Death Domain (TRADD), Receptor Interaction Protein 1 (RIP1) kinase, ubiquitin ligases TNFR-Associated Factor 2 (TRAF2) and cellular Inhibitor of Apoptosis 1 and 2
(c-IAP1 and c-IAP2) to form a regulatory complex that has implications in either cell death or inflammatory responses such as involvement of NF-κB signaling [114, 117].

The intrinsic apoptotic pathway is activated in a cell-autonomous manner upon cellular stress signals via the mitochondria (Figure 1.3). Developmental or stress-induced signals initiate Mitochondrial Outer Membrane Permeabilization (MOMP) to release factors in the mitochondrial intermembrane space such as cytochrome c, Smac/Diablo or Omi/HtrA2 into the cytosol [119-125]. MOMP is believed to display a point of no return in apoptosis and it is a tightly regulated process by a family of B-cell Lymphoma 2 (Bcl-2) proteins [126-129]. Bcl2 family proteins are defined by having at least one Bcl-2 Homology (BH) motif that determines pro- or anti-apoptotic function of the protein [130]. There are three classes of Bcl-2 family proteins. The first class is pro-apoptotic effector proteins such as BAX, BAK and BOK that regulate MOMP directly [131]. The second class involves pro-survival proteins such as Bcl-2 and Bcl-xL [130, 132]. The third class consists of pro-apoptotic proteins such as Bid, Bim, Noxa and PUMA that are called BH3-only proteins and can activate effector proteins (BAX/BAK/BOK) or inhibit anti-apoptotic proteins (Bcl-2 etc.) [131].

In dying cells, BAX and BAK function redundantly and are activated through multiple steps including conformational changes, insertion into the Outer Mitochondrial Membrane (OMM), homo-dimerization and higher-order oligomerization, which results in MOMP [131]. BOK is not redundant with BAX/BAK and can mediate MOMP by itself [131]. However, its structural mechanism of activation is less clear. BH3 only
proteins such as Bid and Bim can interact with BAK and BAX, respectively, to initiate their activation [131, 133]. For MOMP to proceed, pro-survival Bcl2 proteins such as Bcl2 and Bcl-xL are inhibited by BH3-only proteins such as Noxa and PUMA [130]. As regulators of both first and second class Bcl2 proteins, BH3-only proteins are tightly controlled at both transcriptional and post-translational levels such as cleavage or modification upon apoptosis induction.

Release of cytochrome c after MOMP is critical for activation of the Apaf1 apoptosome, which is a ring-like cell death platform and analogous to the Dark apoptosome in Drosophila [114]. Cytochrome c binds to auto-inhibited, monomeric and ADP-bound Apoptotic protease-activating factor 1 (Apaf1) [134]. This interaction triggers nucleotide exchange (replacement of ADP to (d)ATP), which results in conformational changes and formation of an active, heptameric apoptosome complex [134]. Apaf1 is a large protein consisting of N-terminal Caspase Recruitment Domain (CARD) that recruits caspase-9 through homotypic interactions between their CARD domains. In addition, Apaf-1 consists of a number of regulatory domains such as Nucleotide Binding Domain (NBD), 2 Helical Domains (HD1, HD2), Winged Helix Domain (WHD) and 15 WD40 repeats. These regions play roles in Apaf1 auto-inhibition, nucleotide exchange and oligomerization [134].

Recent structural studies suggest that the CARD domain of Apaf1 is surface-exposed, dynamic and sits at the spoke of a central hub that consists of regulatory regions, which gives Apaf1 apoptosome a ring/wheel like structural look [135, 136]. The
recruitment of caspase-9 into the Apaf1 apoptosome occurs in 1:1 protein stoichiometry [135]. This recruitment is essential for caspase-9 activation. In the apoptosome, caspase-9 is dimerized and auto-cleaved between its large and small subunits. Auto-cleaved caspase-9 can be released into the cytoplasm to cleave effector caspases caspase-3 and caspase-7 for execution of apoptosis. The released caspase-9 can be replaced with inactive zymogen in the apoptosome to continue efficient processing and activation of caspase-9 [137].

In addition to caspase-8 and caspase-9, mammals have another initiator caspase named caspase-2. Caspase-2 is highly conserved and has a CARD domain at its N-terminus. Similar to other initiator caspases, caspase-2 is induced by dimerization followed by auto-cleavage and recruited into a cell death platform called PIDDosome [138, 139]. This protein complex consists of caspase-2, adaptor protein RAIDD and PIDD. Upon stressed-induced conditions, caspase-2 forms a homotypic interaction with the CARD domain of RAIDD and PIDD binds to RAIDD through their DDs after undergoing sequential cleavage [138, 139]. A recent study suggests that RAIDD forms in a closed state and can only interact with caspase-2 after RAIDD interacts with PIDD, suggesting that PIDD mediates and stabilizes the PIDDosome. [140]. Once assembled, PIDDosome have 7 caspase-2, 7 RAIDD and 5 PIDD proteins [138, 139]. In contrast to other initiator caspases, caspase-2 does not cleave effector caspases but cleaves other substrates that have roles in cell cycle progression, in response to ER-stress, DNA-damage, etc. One reported caspase-2 target is Bid, an interaction that results in MOMP and cytochrome c release [141]. Although caspase-8 and caspase-9 knockout mice are
embryonic lethal, caspase-2 knockout mice develop normally, suggesting that caspase-2 may not be essential for embryonic development [138].

Caspases play essential roles in initiating and executing mammalian apoptosis, similar to flies. Activity of caspases is tightly controlled by a group of Inhibitor of Apoptosis (IAP) proteins. Examples of well-characterized mammalian IAPs are X-chromosome linked IAP (XIAP), cellular IAP1 and IAP2 (c-IAP1, c-IAP2). XIAP has three BIR domains, each having different affinities for caspases/pro-apoptotic proteins (IAP antagonists). While BIR2 of XIAP binds to processed caspase-3 and caspase-7, BIR3 of XIAP binds to processed caspase-9 through their IAP Binding Motif (IBM). IAP antagonists such as Smac/DIABLO and Omi/HtrA2 are released after MOMP and can also bind to both BIR2 and BIR3 domains of XIAP through their IBM. The interaction of XIAP with caspases leads to ubiquitylation and inhibition of caspases, whereas interaction between XIAP and IAP antagonists causes auto-inhibition of XIAP through degradation. XIap knockout mice surprisingly showed no embryonic lethality and developmental defects [142-145]. RING domain deletion of XIAP in mice stabilized XIAP protein and also did not show any embryonic lethality and developmental abnormalities. However, Xiap\textsuperscript{ARing} MEFs and mouse embryonic stem cells showed increased caspase-3 activity and impaired ubiquitination of caspase-3 [146]. Xiap\textsuperscript{ARing} MEFs were also sensitized to TNF-induced apoptosis. Furthermore, Xiap deficient MEFs displayed elevated protein levels of cIAP1 and cIAP2 [142]. This may suggest that other IAP proteins may compensate for the loss of XIAP, resulting in lack of observed phenotypes in development of Xiap knockout mice.
As an example of crosstalk between extrinsic and intrinsic apoptotic pathways, after activation in the DISC, caspase-8 can cleave BH3-only protein Bid. Cleaved Bid can locate to the mitochondria to activate BAX and BAK, which promotes MOMP and release of IAP antagonists [114]. This is a defense mechanism against XIAP inhibition of effector caspases.

C. Apoptosis and Cancer

There is a delicate balance between cell survival and cell death. Even a subtle change could lead to an imbalance of either side. Down-or up-regulation of apoptosis could result in cancer and neurodegenerative diseases. After an extensive introduction to the apoptotic pathway in Part IA and IB, this section will examine various apoptotic players in terms of their contribution to cancer.

Cancer is currently one of leading causes of death in the United States. Downregulation of apoptosis is a hallmark for cancer. Suppression of apoptosis contributes to tumor initiation, progression, metastasis and resistance to therapeutic approaches. Bcl2 family proteins, the upstream regulators of MOMP, play an important role in cancer development. For example, pro-survival Bcl2 gene was found be translocated (t(14;18)) in non-Hodgkin’s Lymphomas, even before its involvement in apoptosis was discovered [147]. This chromosomal translocation results in elevated Bcl2 gene expression. Moreover, downregulation of micro RNAs that repress Bcl2 gene and hypomethylation of Bcl2 were shown to increase Bcl2 gene expression in chronic lymphocytic leukemia [148]. Furthermore, pro-apoptotic Bcl2 family proteins such as
BAX and BAK are misregulated in colon and gastric cancer development [147]. In addition to BAX, BH3-only pro-apoptotic proteins such as Bid, Noxa and Puma are shown to be regulated by P53 through both transcriptional and post-translational levels in cancer cells [148].

Pro-survival Bcl2 and pro-apoptotic BH3-only proteins are important determinants of cancer therapy. Bcl2 proteins are found to protect cells in response to cytotoxic anti-cancer drugs [149]. On the other hand, upstream of Bcl2 proteins, BH3-only proteins are shown to induce apoptosis in response anti-cancer drugs [149]. Recent studies are focused on identifying BH3-only protein mimetics to inhibit pro-survival factors and induce apoptosis of cancer cells.

Death receptors of the extrinsic apoptotic pathway are shown to have implications in various cancer types such as colon, ovarian, cervical and pancreatic cancers [150]. For example, increased expression of antagonistic TRAIL receptors can inhibit TRAIL signaling, contributing to cancer progression. In addition to death receptors, the extrinsic apoptotic pathway can be impaired by low level expression of caspase-8 due to epigenetic regulation [150]. Recent studies suggested that this kind of impairment could be targeted by demethylation agents or HDAC inhibitors for therapeutic reasons. Moreover, caspase-8 activation is regulated through post-translational modifications such as phosphorylation in cancer cells [150].

High level expression of IAPs such as XIAP, cIAP-1, cIAP-2 has been indicated in breast, colorectal, non-small cell lung cancer, cervical cancer and others [151].
Elevated expression of IAPs contributes to cancer through inhibiting apoptosis and ensuring survival of cancer cells. For example, to restrain XIAP activity, Smac mimetics are widely used for therapeutic reasons. Smac mimetics are designed to bind BIR2 and BIR3 domains of XIAP for better activation of caspases [151].

Apoptosome components could be deregulated in cancer. Due to epigenetic regulation, Apaf1 expression level was shown to be downregulated in melanomas, leukemias, glioblastomas and cervical carcinomas [152]. To overcome this dysregulation, pan-histone deacetylase inhibitors could be used to induce expression of Apaf1. In addition, defects in cytochrome c-induced caspase activation were observed in acute myeloid leukemia. As a resistance mechanism to apoptosis, Apaf1 was also shown to be sequestered from the cytosol to plasma membrane in B-cell lymphoma cells. Recent studies are focused on developing cytochrome c mimetics or agents that induce release of cytochrome c from mitochondria for therapeutic reasons. The other component of the apoptosome complex is caspase-9. Caspase-9 is not frequently mutated in human cancers; however it can contribute to tumor progression in sensitized backgrounds [153]. In addition, short isoforms of caspase-9 zymogen were shown to act as dominant negative in tumor cells [154]. In a recent study, germline genetic variations of caspase-9 at both gene and SNP level were observed in non-Hodgkin’s Lymphoma [155]. Therefore, a better understanding of the structural mechanism of apoptosome assembly will help designing drugs to induce apoptosome formation in cancer cells. Although being downstream effectors of the apoptotic pathway, caspase-3 and caspase-7 also play a role in cancer development. For example, caspase-3 transcript and protein levels were downregulated in
primary breast tumor cells [153]. Moreover, mutations in caspase-7 were observed in colon, esophageal and head/neck carcinomas [153].

As examples above show, pro- or anti-apoptotic factors can be misregulated at transcriptional and/or translational level in cancer cells. Misregulation of apoptotic players could contribute to tumor initiation and/or progression. Understanding the apoptotic pathway in more detail will shed light into better therapeutic approaches to overcome cancer.

D. Non-Apoptotic Functions of Caspases

Aside from their jobs to execute cell death and dismantle cells, caspases function in non-apoptotic ways without causing cell death but helping cell differentiation, proliferation, regeneration, etc. This section highlights examples of non-apoptotic functions of caspases in mammalian and Drosophila development.

D1. Non-Apoptotic Functions of Mammalian Caspases

The importance of non-apoptotic functions of mammalian caspases was unveiled through caspase knock out studies. For example, caspase-8 null MEFs showed a deficiency in cell motility due to impairment of activation of calpains, Rac and lamellipodial assembly [156]. Interestingly, loss of caspase-8 was observed in metastatic neuroblastoma [157]. Reconstitution of caspase-8 in neuroblastoma resulted in suppression of their metastasis, suggesting a pro-survival role for caspase-8 in tumor invasion [157]. Caspase-8 was also indicated to be a regulator in NF-κB signaling during
macrophage differentiation [158]. Patients with homozygous deficiency at caspase-8 displayed immunodeficiency dependent on lack of activation of their T-lymphocytes, B-lymphocytes and natural killer cells [159]. In addition, deletion of caspase-8 in T-cells resulted in immunodeficiency, indicating importance of caspase-8 for T-cell homeostasis and T-cell mediated immunity [160].

An interesting study suggested caspase-8 and caspase-3 are required for mediating programming of induced Pluripotent Stem Cells (iPSC) from fibroblast [161]. Both caspases were activated by Oct-4 transcription factor and caspase-3 could cleave Retinoblastoma susceptibility (Rb), a cell cycle regulator, to facilitate iPSC induction [161]. Activity of caspase-3 was also required in Embryonic Stem Cells through cleavage of Nanog and Hematopoetic Stem Cell differentiation due to alterations on cytokine-mediated signaling pathways [162, 163]. Caspase-3 has been implicated in cell proliferation by targeting cell cycle regulators. For example, caspase-3 can promote proliferation of B-lymphoid cells through cleaving Cyclin Dependent Kinase (CDK) inhibitor p27 [164]. An anti-proliferative role of caspase-3 in B-cell homeostasis was described through cleavage of PCDNA interaction domain CDK inhibitor 21 and thus release of PCDNA and impairment of proliferation. Caspase-3 knock-out mice showed defects in muscle cell differentiation. Sterile twenty-like kinase (MST1) cleavage by caspase-3 is required for skeletal muscle differentiation [165]. Activation of caspase-3 was also indicated in keratinocyte differentiation and osteogenic differentiation of bone marrow stromal stem cells [166, 167]. Caspase-3 was also shown to be crucial for neuronal development. For example, active caspase-3 was required for differentiation of
neurospheres, Bergmann glia and PC12 cells. Interestingly, caspase-3, 7, 6 or 8 could cleave Tau, which contributes to Alzheimer’s Disease due to enhanced assembly of cleaved Tau (discussed in Part IC2) [168]. In addition, a recent study uncovered that caspase-2 could cleave Tau, which does not trigger its fibrillation but regulate mis-sorting of full-length Tau [169]. This cleavage product was elevated in Alzheimer’s Disease cases and decreasing levels of caspase-2 could protect against neurodegeneration [169].

In addition to caspase-3, the initiator caspase-9 was implicated in neuronal development. Activity of caspase-9 was found to be necessary for maturation of olfactory neurons during late embryogenesis [170]. In particular, caspase-9 cleaved Semaphorin7A, a member of membrane-anchored Semaphorin family proteins for axonal guidance [170]. Caspase-9 and caspase3/7 were also shown to be involved in Long Term Depression (LTD) and AMPA receptor internalization in hippocampal neurons through cleavage and inactivation of Akt1 which was shown to regulate synaptic modifications via LTD [171]. Caspase-9 and caspase-3 were also indicated to play a role in axonal degeneration and pruning [172]. Specifically, Neural Growth Factor (NGF)-deprived neurons exhibited high activity of these caspases [172].

Caspases can also function in response to inflammation. Human caspases-1, -4, -5 and -12 that carry CARD domains were found to be mediating inflammatory responses [173, 174]. In particular, caspase-1 can be recruited to distinct protein complexes called inflammasomes upon external or internal insults such as infections, toxins, metabolites and irritants [173, 174]. Caspase-1 can be activated by the inflammasome formation,
which leads to cleavage and maturation of Interleukin (IL) cytokine family members IL-1β and IL-18 that are important for inflammatory responses [173, 174]. A distinct form of cell death, pyroptosis can also be initiated by caspase-1 activation through the inflammasome in macrophages and dendritic cells upon infection by intracellular pathogens [175]. Pyroptosis has different morphological features compared to apoptosis and downstream effectors of caspase-1 in pyroptotic cell death are still not well-known [175].

**D2. Non-Apoptotic Functions of Drosophila Caspases**

While apoptotic stimuli cause cell death autonomously, it can also signal non-autonomously to proliferate and compensate for the loss of dying cells. This is called ‘Apoptosis induced Proliferation’ (AiP). This phenomenon was first described forty years ago in a study involving *Drosophila* imaginal discs and their regenerative capacity upon bisecting or grafting operations [176]. *Drosophila* imaginal discs that are composed of epithelial monolayer are great tools to study regeneration due to their remarkable regenerative capacity. One popular model for regeneration is overgrowth associated by the co-expression of *hid* or *rpr* and *p35* in the *Drosophila* eye or wing imaginal discs. Expression of *hid* or *rpr* in the *Drosophila* eye induces apoptosis; however, simultaneous expression of *p35* blocks the execution of apoptosis, which results in overgrowth of the head capsule, multiplication of bristles and ocelli. This model system is used to identify components of AiP. Earlier studies showed that Dronc activity is necessary and sufficient for AiP [177, 178]. Wingless (Wg) and Decapentaplegic (Dpp) signaling were activated
in another setting of AiP when cell death is induced through X-ray or heat shock and blocked with P35 expression [179]. The importance of mitogenic signaling and activation of Jun N-terminal Kinase (JNK) was depicted in another report, which placed wg epistatic to JNK signaling [180]. EGFR signaling was also shown to be activated downstream of JNK signaling in AiP [181]. The link between Dronc and JNK signaling in AiP came from a recent study in which our lab showed that Drosophila macrophages are activated by extracellular Reactive Oxygen Species (ROS) and induce JNK signaling through release of Drosophila TNF, Eiger and its receptor Grindelwald [182]. Signaling roles for Dronc were uncovered in another study that showed that Dronc could induce Drosophila p53 (dp53) for cell-cycle arrest and AiP [183]. Further examination of the role of dp53 uncovered that N-terminally truncated form of dp53 activates Wg signaling to promote AiP [184]. Another type of AiP is indicated in differentiating tissue as opposed to proliferating tissue where most of the studies are done. This particular study showed that effector caspases turn on Hedgehog signaling in photoreceptor neurons expressing hid, which triggers proliferation of undifferentiated yet post-mitotic cells [185].

A few non-apoptotic functions of caspases could be detected in cell differentiation such as patterning of arista and bristles. Drosophila antenna has a feather-like structure at its tip, called arista. Arista development in Drosophila was abnormal when hid, diap1, dronc, drice or dcp-1 were not functional [66, 186, 187]. Interestingly, the abnormal arista phenotype was not observed when P35 was overexpressed [66, 186, 187]. Null alleles of a myosin family factor crinkled (an interactor of Dronc) also exhibited
abnormal arista development [188]. However, it is not clear how caspases could regulate this process in a non-apoptotic way. Mechanosensory bristles in adult *Drosophila* thorax originates from Sensory Organ Precursors (SOP) in the larval wing discs through a series of cell divisions. Loss-of-function mutants of *dark, dronc, ubcd1* or expression of a dominant negative *dronc* mutant showed extra bristles on the notum, suggesting activation of Dronc is essential in this process, dependent on Dark [71, 72, 82]. Further examination of this phenotype revealed Shaggy46, an isoform of *Drosophila* GSK-3/Shaggy, as a caspase substrate [189]. Shaggy is cleaved by caspases to get activated and antagonize Wg signaling in SOP development [189]. A recent study suggested that CRINKLED could recruit Shaggy46 to Dronc to mediate cleavage of Shaggy46 [188]. In the absence of caspase activity, an additional SOP cell differentiates and gives rise to an extra bristle. Interestingly, *Drosophila* IKKε could phosphorylate DIAP1, leading to its degradation in proneural cells in wing discs, which controls caspase activation in SOP cell development [190]. In line with these findings, loss of one functional copy of *diap1* suppressed the extra bristle phenotype [191].

Caspases are also found to play a role in neural pruning. To sculpt neural circuits, larval axons such as Class IV dendritic arborization (C4da) neurons undergo pruning during metamorphosis. Caspase activity was localized in C4da neuron dendrites and observed at late stages of pruning [192]. Ubcd1 and Valocin-containing protein (VCP), a ubiquitin-selective AAA chaperone, contributed to degradation of DIAP1 to control activation of Dronc in C4da dendrites [193, 194]. Additionally, mutations in *dronc*
strongly suppressed pruning, while mutations in *drice* suppressed pruning mildly and mutations in *dcp-1* had a weak suppression [195].

Caspases are implicated in immune responses. In *Drosophila*, the caspase-8 homolog Dredd was shown to regulate NF-κB signaling. Upon immune stimulation Dredd could cleave IMD, which exposes an N-terminal IBM for DIAP2 interaction, and NF-κB factor Relish for its activation (discussed in Chapter I, Part IIIB) [196-199].

An interesting study in *Drosophila* neuroblasts suggested a role for Dronc that is independent of its catalytic activity. *Drosophila* neural stem cell (neuroblast) homeostasis is controlled by Numb which limits proliferative potential of neuroblasts. Phosphorylation of Numb negatively regulates its activity because phospho-mimetic Numb induced excess neuroblast production. Dronc was identified to interact with wild type or phospho-mutant Numb better than with phospho-mimetic Numb [200]. In addition, overexpression of wild type or catalytically inactive Dronc could rescue proliferative phenotype of neuroblasts associated with phospho-mimetic Numb expression, suggesting a non-apoptotic role for Dronc in regulating neuroblast homeostasis through acting on Numb [200]. It will be interesting to show how interaction of Dronc with Numb could exert this regulation and whether a non-catalytic function of Dronc is also required for homeostasis of other types of stem cells.

An example for regulation of cell migration through non-apoptotic function of caspases is depicted with border cell migration in the *Drosophila* ovary. A group of follicle cells migrate to the center of the egg chamber during *Drosophila* oogenesis [201].
This phenomenon is controlled by various molecular players such as Rac GTPase [202]. Expression of a dominant negative form of Rac (RacN17) in border cells blocks the migration, which could be suppressed by expression of DIAP1 independently of its anti-apoptotic function [203]. This suppression required both BIR domains but not the RING domain [202]. In fact, DIAP1 could physically interact with Rac in vitro [203]. Interestingly, loss-of-function of dark or dominant negative form of dronc could also suppress this blockage of border cell migration, suggesting that Dronc may play a negative role in border cell migration [203]. Supportive of this result, another study showed that the kinase IKKe that could lead to degradation of DIAP1 could also inhibit border cell migration when overexpressed [204]. Interestingly, a recent study indicated that a dominant negative form of crinkled could also suppress RacN17-induced phenotype [188].

Spermatid individualization occurs late in spermatogenesis. 64-interconnected spermatids go along in a cyst while they get rid of their cytoplasm and organelles and mature to become individual spermatids that are enclosed with plasma membrane. Both initiator and effector caspase activity and Dark are required for elimination of these extra structures during spermatid individualization [103, 205]. In addition, male germline specific cyto-c-d is essential for this process because it is required for activation of caspases [103, 105, 205]. Caspase activity is regulated by a Cullin3 ubiquitin ligase complex which targets DIAP1 and dBruce [103, 206-208]. The activity of Cullin3 complex is controlled by its antagonist Soti which competes with substrates or its activator A-Sβ (a Krebs cycle component), which interacts with Cullin3 complex at the
surface of mitochondria [103, 206-208]. This regulation is dependent on spatial and temporal expression of these components during the individualization process [103, 206-208]. Another study identified that Tango7 genetically and physically interacts with the apoptosome to transform its apoptotic activity into a cell remodeling one during spermatid individualization [209].

As seen in examples of non-apoptotic functions of caspases above, there are possible mechanisms how caspase activity could be used in cellular events other than cell death. One of these mechanisms could be localizing caspases in specific subcellular compartments such as in spermatid individualization or neural pruning. Another mechanism could be fine-tuning the levels of caspases through signaling events upstream of caspases such as in SOP cell development. Although it has been more than ten years since the first observations of non-apoptotic functions of caspases, it will be intriguing to discover new regulatory roles for caspases and understand the mechanisms underlying these functions.

**Part II. The Ubiquitin System**

Organisms require a proper communication system to translate cues into action. One such mechanism is the ubiquitin system that ensures homeostasis of the organism through controlling cellular events such as division, differentiation and survival. The fate of a protein and thus regulation of such cellular events can be determined by the ubiquitin system. Conjugation of a 76 amino acid molecule called ubiquitin (Ub) to a substrate
(ubiquitylation) can result in either its proteasomal degradation or change in its localization, activity, conformation or interaction with its binding partners (Figure 1.5) [210].

Ubiquitylation is a post-translational modification. The mechanism of ubiquitylation requires an Ub, an E1 Ub-activating enzyme, an E2 Ub-conjugating enzyme and an E3 Ub-ligase (Figure 1.4). In an ubiquitylation reaction, Ub is first activated in an ATP-dependent manner by joining the carboxyl group of Gly76 of Ub to the catalytic Cys of the E1 enzyme through a thioester bond [211, 212]. Activated Ub is then transferred to catalytic cysteine of the E2 enzyme and forms an E2-Ub thioester [211, 212]. The E3 enzyme binds to both E2-Ub and the substrate by regulating E2 and substrate specificity [211, 212]. The E3 enzyme, depending on its structure either transfers Ub from the E2 to the substrate (RING E3s) or transfers Ub to its catalytic Cys first and then to the substrate (HECT E3s) [211, 212]. In the end, the carboxyl group of Ub (Gly76) forms an isopeptide bond with the ε-amino group in the side chain of a Lys in the substrate. This is called mono-ubiquitylation of a substrate. Proteins can also be modified at multiple Lys residues (multi-mono-ubiquitylation). Mono-ubiquitylation of a substrate is often linked to non-degradative means of regulation (See below for detail).

Ubiquitin is a stable protein that is conserved from yeast to human. It has seven Lys residues: K6, K11, K27, K29, K33, K48 and K63. After a substrate is mono-ubiquitylated, any of these Lys residues or the first Methionine (Met1) can be used for addition of another ubiquitin molecule to form poly-ubiquitin chains. This chain
formation can occur between the same type of linkage (homotypic) or different type of linkages (such as mixed or branched chains). Poly-ubiquitylation of a substrate could lead to either its proteasomal degradation or its non-degradative regulation (See below for details). Poly-ubiquitin chain specificity is determined by E2 enzymes, whereas substrate specificity is controlled by E3 ligases.

Regulation of ubiquitylated proteins often requires interaction with other proteins to fulfill their fate. For example, a substrate to undergo degradation is poly-ubiquitylated and recruited to the proteasome in multiple steps. One of these steps includes the interaction between ubiquitin and a Ubiquitin Binding Domain (UBD) containing protein or between ubiquitin and the proteasome. For this reason, ubiquitin has hydrophobic surfaces such as the Ile44 Patch with Leu8, Val70 and His68 and the Ile36 Patch with Leu8, Leu71 and Leu73 [210]. The Ile44 Patch can mediate interactions with the proteasome and UBDs, while the Ile36 Patch can be bound by UBDs and Deubiquitylating enzymes (DUB) [210].

Ubiquitylation is a reversible process. Deubiquitinases (DUBs) hydrolyze the isopeptide bond between ubiquitin and the substrate or between ubiquitin molecules [213]. DUBs are important for recycling and maintaining free ubiquitin in the cells by removing ubiquitin from proteins that will undergo degradation [213]. They also break bonds between un-anchored linear ubiquitin chains to generate mono-ubiquitin that will be used for ubiquitylation of a substrate [213].
The ubiquitin system utilizes an Ubiquitin, ATP, an E1 activation enzyme, an E2 conjugating enzyme and an E3 ligase. Ubiquitin is conjugated to a Lysine residue of a substrate, which is called mono-ubiquitylation. Addition of ubiquitin to Lysine residues of the ubiquitin on the substrate results in poly-ubiquitylation. Ubiquitylation could regulate substrates in cellular events through non-degradative or degradative manners and can be reversed by deubiquitinases.
In addition, they function in regulating ubiquitylated proteins through chain editing and by acting as antagonists for ubiquitylation [213].

Ubiquitylation is a reversible process. Deubiquitinases (DUBs) hydrolyze the isopeptide bond between ubiquitin and the substrate or between ubiquitin molecules [213]. DUBs are important for recycling and maintaining free ubiquitin in the cells by removing ubiquitin from proteins that will undergo degradation [213]. They also break bonds between un-anchored linear ubiquitin chains to generate mono-ubiquitin that will be used for ubiquitylation of a substrate [213]. In addition, they function in regulating ubiquitylated proteins through chain editing and by acting as antagonists for ubiquitylation [213].

A. Degradative Ubiquitylation

The Ubiquitin-Proteasome System (UPS) was first described more than 30 years ago by Avram Hershko and his colleagues, which had earned them the Nobel Prize in Chemistry in 2004 [214-216]. Cells utilize UPS to selectively get rid of misfolded and abnormal proteins [212]. In addition, the UPS controls concentrations of short-lived proteins and contributes to regulation of protein activity by destroying inhibitory regions [212]. One of the most-studied and abundant types of ubiquitylation is K48 poly-ubiquitylation. K48 linkage with four or more ubiquitins targets substrates to the 26S proteasome for degradation [210]. NMR studies of tetra ubiquitin chains revealed that K48 poly-ubiquitin chains adopt a compact conformation by interaction of hydrophobic moieties with each other, thus limiting its surface exposure for regulatory reasons [210].
A recent study conducted in yeast showed that upon proteasome inhibition, K11 linkage was found to be the most abundant after K48 and followed by K63, K6 and K29 linkages, suggesting that non-K48 linkages also contribute to UPS [217]. Studies focusing on K11 linkage identified its role in the cell cycle. Induction of Anaphase Promoting Complex (APC/C), which mediates degradation of cell cycle proteins, led to accumulation of K11 signal and inhibition of K11 linkage resulted in stabilization of APC/C targets [218, 219]. Branched poly-ubiquitin chains were also shown to be involved in UPS. For example, branching of K11 ubiquitin chain with K48 and K63 can be synthesized by APC/C to function in and improve proteasomal degradation [220]. As being one of most well-known regulatory poly-ubiquitylation signal, K63 linkage was also reported to contribute to UPS in yeast [221]. Interestingly, a recent study indicated that upon inhibition of formation of poly-ubiquitin chains, mono-ubiquitylated proteins can undergo proteasomal degradation in yeast and mammalian cells [222].

Linkage specificity is not a random process. E2 conjugating enzymes determine length and topology of a ubiquitin chain [223]. By controlling chain initiation and elongation, one E2 enzyme can bind to multiple E3 ligases to regulate their substrates’ fate [223]. For example, APC/C conjugates K11 poly-ubiquitin chain with the help of Ube2S [224]. Linkage specificity for different DUBs has also been shown before. For example, OTUB1 and Cezanne, two members of the Ovarian Tumor (OTU) family of DUBs, specifically remove K48 linkages and K11 linkages, respectively [225-227].

**B. Non-Degradative Ubiquitylation**
In addition to degradation, ubiquitylation of a substrate can also regulate its activity, interactions and localization. Non-degradative ubiquitylation can occur through mono-ubiquitylation or poly-ubiquitylation of substrates [210]. Regulation of protein interactions through mono-ubiquitylation has been implicated in DNA damage response [210, 228]. For example, upon DNA damage, PCNA (a processivity factor for DNA polymerases) is mono-ubiquitylated [210, 228]. This recruits Y family DNA polymerases that substitute for replicative polymerases from PCNA and restore replication process [210, 228]. In addition, FANCD2 and FANCI are found to be mono-ubiquitylated, which leads to their assembly in nuclear foci together with other related proteins for DNA repair [229]. Both of these mono-ubiquitylation events can be reversed by Usp1 deubiquitinase [228]. Another substrate of mono-ubiquitylation that affects protein interaction is Smad4, central transducer of TGFβ signaling. Mono-ubiquitylation of Smad4 blocks its interaction with Smad2/3 and thus hinders formation of an active complex and represses its transcriptional targets [230]. In the case of P53 regulation, mono-ubiquitylation induces its transportation from nucleus to cytoplasm, resulting in inhibition of its target genes [231].

K63 poly-ubiquitylation is the most commonly observed non-degradative poly-ubiquitylation. NMR analysis and crystal structure of K63 poly-ubiquitin chain displayed an open conformation, which could support chain flexibility and distance for diverse regulation [210]. K63 poly-ubiquitylation and Met1- linked linear ubiquitin chains have regulatory implications in NF-κB signaling (see Part III for details) [210, 232].
The ubiquitin system has implications in both degradative and non-degradative means of regulation of proteins in cellular events as mentioned above. It is important to understand the relationship between the ubiquitin system and apoptosis as both pathways regulate cell homeostasis. The following section will discuss examples of how the ubiquitin system controls apoptosis and what are the consequences of ubiquitylation of anti- and pro-apoptotic proteins in detail.

Part III. Regulation of Apoptosis through the Ubiquitin System

A. The Ubiquitin System and Mammalian Apoptosis

Inhibitor of apoptosis proteins (IAPs) are important for the cells’ defense against caspase activity. The physiological inhibitor of caspases in mammals is XIAP, which is shown to target caspase-3, 7 and 9 for ubiquitylation and thus inhibition (discussed in Part IB). IAP antagonist such as SMAC/DIABLO, HtrA2/Omi and ARTS were shown to be ubiquitylated and degraded by XIAP [233].

The mature form of caspase-9 could interact with BIR3 of XIAP through its IBM (residues 316-319) [234]. A structural study revealed that both mature and pro-caspase-9 are monomers in solution; however, interaction of mature caspase-9 with XIAP prevents caspase-9 to homodimerize [235]. This interaction also masks the activation interface of caspase-9 so that caspase-9 function is fully inhibited [235]. In the case of effector caspases, the linker region between BIR1 and BIR2 of XIAP binds to caspase-3 or 7
active homodimers and hinders the substrate entry for cleavage [236-238]. Studies conducted in mammalian cells suggested that XIAP could ubiquitylate caspase-3 for degradation [239]. Mature caspase-9 was also shown to be poly-ubiquitylated by XIAP at its large subunit for degradation [240]. However, deletion of the RING domain of XIAP in mice did not result in increase in protein levels of caspase-3, 7 or 9 [146]. This could suggest that these caspases could be regulated by the ubiquitin system in a non-degradative way.

An interesting study in rat brains showed that XIAP could be mono-ubiquitylated to regulate its localization and/or activity upon traumatic brain injury [241]. However, whether XIAP or another ubiquitin ligase mediates this mono-ubiquitylation is unclear.

In addition to XIAP, mammalians have c-IAP1 and c-IAP2 E3 ligases that function in the extrinsic apoptotic pathway. c-IAP1 and c-IAP2 bear three BIR domains, a Ubiquitin-Associated (UBA) domain, a CARD and a RING domain [242]. They do not inhibit caspase-8 directly, however, they are important signaling proteins in TNF-induced cell death. c-Iap1 and c-Iap2 double knockout Mouse Embryonic Fibroblast (MEF) cells are resistant to TNF-induced cell death [242]. Upon infection, TNFα signaling could be activated as a response in three distinct mechanisms, which could lead to survival, apoptosis or necrosis (another type of cell death) [243]. Binding of TNFα to TNFR1 recruits TRADD which then acts a scaffolding protein to recruit RIP1 and ubiquitin ligases TRAF2, c-IAP1 and c-IAP2 and form Complex I [243]. In this protein complex, c-IAP1 and c-IAP2 partner with UBC5/UBE2D1 E2 enzyme to ubiquitylate RIP1,
TRAF2 and themselves non-degradatively [243]. The complex formation followed by these ubiquitylation events culminate in activation of NF-κB signaling [243]. Linear Ubiquitin Assembly Complex (LUBAC) could also ubiquitylate RIP1 linearly to further stabilize TNFR signaling [242]. If RIP1 ubiquitylation is inhibited by blockage of c-IAPs or removed by deubiquitinases such as cylindromatosis (CYLD), RIP1 and TRADD are released from the Complex I [242]. Free TRADD monomer could interact with FADD through Death Domain interaction [242]. FADD then binds to Caspase-8 through the Death Effector Domains [242]. These interactions form a complex for caspase-8 dimerization and activation (Complex II), which leads to apoptotic signaling [242]. If caspase-8 is inhibited, RIP1 binds to RIP3, which mediates necroptotic cell death [243]. Therefore, TNF signaling nicely illustrates the importance of the ubiquitin pathway in recruitment and assembly of signaling complexes.

The function of the CARD domain in an IAP is still a mystery. However, a recent study highlighted that the CARD of c-IAP1 could inhibit the E3 ligase activity of c-IAP1 through preventing RING dimerization which is a critical step for activation of c-IAP1 and c-IAP2 [244]. This inhibition is important to block caspase-8-mediated apoptosis through TNF signaling [244].

The UBA domain of c-IAP1 was shown to bind to mono-ubiquitin and K48- and K63-linked poly-ubiquitin chains, which serves a scaffolding platform for NF-κB signaling [245, 246]. The UBA domain of c-IAP1 and c-IAP2 are required for their anti-apoptotic function; however, mutations in UBA did not abolish the recruitment of TNF
signaling components [245]. Similar to XIAP, IAP antagonists can also bind to BIR3 domain of c-IAP1 and c-IAP2, which leads to their auto-degradation. Interestingly, the UBA domain was necessary for IAP antagonist-induced proteasomal degradation of c-IAP1 and c-IAP2 [245]. Similar to c-IAP1 and c-IAP2, XIAP also carries a UBA domain that was shown to bind mono-ubiquitin, K48-linked and linear di-ubiquitin chains [247], which could serve as a scaffold for regulatory reasons.

C-IAP1 and 2 were shown to be involved in interaction and ubiquitylation of caspase-3, 7 and SMAC/DIABLO. Interestingly, caspase-3 and 7 were shown to be mono-ubiquitylated by c-IAP2 in vitro [248]. In a recent study, c-IAP1 was shown to bind processed caspase-7 and intermediate form of caspase-3 [249]. These interactions result in ubiquitylation and degradation of caspase-7 and 3 [249]. Another study suggested that c-IAP1 inhibits procaspase-3 but not active and mature caspase-3 in mammalian cells through interaction with the apoptosome [250]. However, compared to XIAP, c-IAP1 is a poor physiological inhibitor of effector caspases [249]. The reason for this observation could be that XIAP, c-IAP1 and c-IAP2 have distinct interaction mechanisms with caspases that may affect consequences of IAP-mediated inhibition [251]. Regulation among IAPs through ubiquitylation could also be possible. For example, c-IAP1 could target XIAP for degradation [233].

Recently, caspase-8 activation was shown to be regulated by ubiquitylation. In the DISC, caspase-8 activation is induced through poly-ubiquitylation (potentially K63-linked) at its small catalytic subunit by Cullin3 E3 ligase [252]. Activated caspase-8
interacts with TRAF2 E3 ligase and undergoes K48-linked poly-ubiquitylation at its large subunit, which results in proteasomal degradation of caspase-8 [253]. These ubiquitylation events elegantly show how activation of caspases could be regulated by the ubiquitin system.

Another interesting study suggested that Apollon E2 enzyme with a BIR domain could poly-ubiquitylate mature or procaspase-9, SMAC/DIABLO and HtrA2/Omi in an E3-independent, but UBC-dependent manner [254-256], which results in proteasomal degradation of these targets.

Neuronal Apoptosis Inhibitory Protein (NAIP) is the first identified E3 ligase with three BIR domains and a Nuclear Oligomerization Domain (NOD). NAIP was originally misidentified as the gene for spinal muscular atrophy [257]. In cell culture studies, the BIR2 and BIR3 domains of NAIP, BIR2 with more potency, could inhibit the function of caspase-3 and caspase-7 [258]. Further studies revealed that NAIP could not interact with SMAC/DIABLO [259, 260]. However, the BIR3 domain of NAIP but not BIR3 of XIAP could interact with procaspase-9 in the apoptosome in an ATP-dependent manner but not with monomeric procaspase-9 [259, 260]. Another study on this observation showed that the NOD of NAIP actually interacts with CARD-NOD of APAF1 [261]. Hence, this regulation of the apoptosome by NAIP inhibits processing of procaspase-9 and thus procaspase-3 [261].
Involvement of SCF type ubiquitin ligase complexes were also shown in mammalian apoptosis. For example, SCF(β-TRCP) could ubiquitylate a pro-apoptotic BCL-2 family protein BIMEL and procaspase-3 for degradation [262, 263].

In addition to IAP E3 ligases, DeUbiquitinating Enzymes (DUB) constitute an important part of the Ubiquitin System. Multiple DUBs are implicated in regulation of apoptosis [264]. For example, USP15 was found to control stability of procaspase-3 positively and its interaction with SCF complex negatively [265]. Similarly, USP41 was shown to be a pro-apoptotic DUB as expression of USP41 activated caspase-3 [266]. DUBs are also shown to regulate apoptosis through signaling at upstream of caspases [264].

**B. The Ubiquitin System and *Drosophila* Apoptosis**

Being an E3 ligase, DIAP1 represents the central link in the hub of apoptosis and ubiquitylation network. DIAP1 could target both RHG proteins and caspases (discussed in detail in Part IA). However, regulation of apoptosis through ubiquitylation is not limited to DIAP1, other players in the ubiquitin pathway have been characterized to regulate *Drosophila* apoptosis via DIAP1 or other players.

Even before *Drosophila* caspases were shown to be regulated non-degradatively by DIAP1, an interesting study found that DIAP1 could generate K63 poly-ubiquitin chain for its auto-ubiquitylation with Ubcd1 or Ubc13/Uev1a as E2 ligases [267]. This K63 poly-ubiquitylation of DIAP1 seems to be blocking its E3 ligase activity on its targets, most likely through steric hindrance [267]. In confirmation with other studies,
this study also showed that DIAP1 is cleaved by caspases at its N-terminus, which makes it a substrate for the N-end rule pathway [267]. However, the same study also uncovered that full-length DIAP1 could be degraded with or without apoptosis induction by a novel E3 ligase, independently of caspases, its E3 ligase activity and N-end rule pathway [267]. DIAP2 was shown to bind and induce degradation of DIAP1 [267]. It is possible that other ligases are also involved in this new type of DIAP1 regulation.

A new look at DIAP1-induced RHG protein ubiquitylation identified that dimerized GRIM could bind to DIAP1 through BIR1 and BIR2 domains; however only BIR2-bound GRIM could be ubiquitylated by DIAP1 at K136 [268]. DIAP1 cooperates with Ubcd1 to conjugate K48-linked poly-ubiquitin chains to GRIM for proteasomal degradation [268]. Interestingly, this ubiquitylation can be prevented when GRIM is cleaved by caspases at Asp132 [268]. A cleavage mutant GRIM was less potent activator of cell death than wild type GRIM in Drosophila S2 cells [268]. One explanation for this phenotype would be that GRIM is in a feed-forward loop where activated caspases cleave GRIM to block its ubiquitylation so that it could continue antagonizing DIAP1 and activating caspases.

A screen to identify E3 ligases in the apoptotic pathway led to characterization of UBR3 [269]. It has been previously shown that UBR proteins are involved in apoptosis such as in DIAP1 degradation through the N-end rule pathway or interacting with DIAP1 for its anti-apoptotic function. This study showed that downregulation of UBR3 in the Drosophila eye caused an abnormal eye phenotype due to increased apoptosis [269].
UBR3-induced cell death appeared to be upstream of Dronc but downstream of or in parallel to DIAP1 [269]. Interestingly, the UBR-box domain but not the RING domain of UBR3 was essential for its anti-apoptotic function [269]. UBR1, which was shown to interact with DIAP1 for its anti-apoptotic function in Drosophila cells together with UBR3, -5, -6, -7, could not suppress UBR3-induced cell death, suggesting that these enzymes have different functions in apoptosis [269]. N-terminally cleaved but not wild type DIAP1 could interact with UBR3 through the UBR-box domain, enhancing its interaction with caspases and thus its ability to ubiquitylate caspases [269].

UBA1, the Drosophila E1 enzyme, was found as a suppressor of hid-induced cell death in the Drosophila eye; which displays a good example of the impact of ubiquitylation to Drosophila apoptosis [270]. Further examination of this phenotype identified that weaker Uba1 alleles could suppress both ectopic and developmental cell death due to a still functioning ubiquitin system [270]. Because the cells contained increased DIAP1 and decreased Dronc levels [270]. In contrast, stronger Uba1 alleles which block the ubiquitin system induced apoptosis as DIAP1 could not utilize the ubiquitin system for inhibiting Dronc [270]. This study also argued that ubiquitylation of DIAP1 versus Dronc occurs at different rates to set the equilibrium of life and death [270].

A recently characterized E3 ligase, elfless has been indicated to induce apoptosis when expressed in the Drosophila eye, which could be suppressed by heterozygosity at dronc or enhanced by heterozygosity at ubcd1 [271]. Extracts from elfless expressed
Drosophila heads showed increased levels of DIAP1 and no change in UBCD1 level [271]. These results may suggest that DIAP1 stability could be regulated by Elfless and UBCD1 in the apoptotic pathway through affecting UBCD1 activity rather than its protein level or degradation of RHG proteins. Further characterization of the function and the nature of elfless will be interesting in terms of finding out its place in the apoptotic pathway.

A screen to identify modifiers of rpr/grim-induced apoptosis in the Drosophila eye characterized four enhancers related to ubiquitylation: the E1 activating enzyme UBA1, the deubiquitylating enzyme fat facets (faf) and two proteins of an SCF E3 ubiquitin ligase complex, Skp1 and Morgue [272]. Heterozygosity at the morgue locus resulted in suppression of cell death induced by rpr/grim, rpr or hid in the Drosophila eye and inhibition of removal of extra interommatidial cells in the Drosophila eye [272, 273]. Expression of morgue in Sf-9 cells caused apoptosis, which could be suppressed by P35 and DIAP1 expression, suggesting that Morgue functions in a caspase-dependent pathway, antagonist to DIAP1 [272, 273]. Morgue contains F-box and E2 conjugating domains [272, 273]. Because F-box and Skp proteins are known to interact with SCF complexes, the authors tested the ability of Morgue and Skp1 to interact [272, 273]. They observed an interaction between these two proteins, dependent on the F-box domain of Morgue [272, 273]. Because Morgue is also an E2 ligase, its interaction with DIAP1 was tested in the same study. DIAP1 and Morgue interacted independent of F-box and E2 conjugating domain of Morgue and the RING domain of DIAP1 [272, 273]. Expression of morgue in S2 cells or 3rd instar larval eye discs led to reduction of DIAP1 level, which
was independent of the RING domain of DIAP1 and could be suppressed by inhibition of the proteasome [272, 273].

dBruce can suppress rpr- and grim-induced cell death in the Drosophila eye [31]. Further examination of this phenotype showed that the levels of a non-ubiquitable RPR are still increased in dBruce mutant background [274]. In fact, RPR is a direct target of dBruce. The BIR domain of dBruce could interact with the IBM and GH3 region of RPR [274]. Interestingly, this interaction provides ubiquitylation of RPR at non-lysine residues [274]. It will be of interest to see if other IAP antagonists are regulated similarly by dBruce.

DeUBiquitylating-Apoptotic-Inhibitor (DUBAI) was characterized to stabilize DIAP1 and protect cells from apoptosis [275]. Downregulation of DUBAI led to eye ablation which was enhanced by rpr-, hid- and grim-induced cell death and was suppressed by DIAP1 in the Drosophila eye [275]. In addition to their genetic interaction, DUBAI and DIAP1 physically interacted and this interaction stabilized DIAP1 [275].

The recently identified Drosophila USP5 deubiquitinase (dmUSP5/leon) was shown to be required for Drosophila development because null allele of dmUsp5/leon are late larval lethal accompanied with increased apoptosis in larval brain and imaginal discs [276]. Extracts prepared from dmUsp5/leon null larva showed that hid and rpr genes were upregulated [276]. Expression of DIAP1 partially rescued the developmental and apoptotic phenotypes [276]. Mammalian USP5 is important for disassembling conjugated or unconjugated poly-ubiquitin chains to mono-ubiquitin [276]. Null mutants of
dmUsp5/leon have decreased or increased free mono-ubiquitin and increased conjugated and unconjugated poly-ubiquitin chains that could impair ubiquitin homeostasis [276, 277]. In particular, dmUSP5/Leon could hydrolyze K48-linked poly-ubiquitin chain better than K63 poly-ubiquitin chain [277]. In fact, dmUsp5/leon null mutant exhibited proteasomal impairment [277]. However, a more recent study showed that dmUSP5/Leon could cleave unanchored K11, K63 and linear poly-ubiquitin chain in addition to K48 [278]. The same study also provided evidence that knockdown of dmUsp5/leon did not result in decrease in mono-ubiquitin levels due to compensation of the function by increased transcription of USP14 [278]. The authors suggested that the function of dmUSP5/Leon is not to maintain mono-ubiquitin levels as opposed to its mammalian counterpart [278]. Apoptosis could be affected through inhibition of proteasomal degradation in dmUsp5/Leon mutants; however, the underlying mechanism is still unclear and yet to be discovered.

Another deubiquitinase, emperor’s thumb(et)/scrawny, was identified to have a role in the apoptotic pathway [279]. Loss of et function resulted in abnormal eye phenotypes in the Drosophila eye, which stemmed from increased apoptosis and reduced DIAP1 level [279]. et has 6 isoforms of which a short and a long isoform have been characterized. Both isoforms have protease activity; however, the short isoforms lack an N-terminal 55 aa sequence [279]. Expression of the long et isoform in the Drosophila eye blocked interommatidial cell death and suppressed rpr- and grim- induced cell death [279]. Interestingly, expression of the short isoform in the Drosophila eye behaved in the opposite way and caused a small eye phenotype due to apoptosis, which could be
partially suppressed by expression of the long et isoform or DIAP1 or Dronc downregulation [279]. However, this phenotype could not be suppressed by P35 expression [279]. It is possible that long and short et isoforms have competing functions to fine-tune levels of apoptotic factors such as RPR, GRIM, DIAP1 and Dronc.

Recently, a deubiquitinase called DUBA was characterized to induce apoptosis. In vitro studies suggested that DUBA interacted with DIAP1 and Dronc to possibly regulate their functions [280]. In case of the mammalian counterpart, phosphorylation of DUBA at S183 was required for its catalytic activity [280]. Interestingly, DUBA could remove K11 and K63 poly-ubiquitin chains more efficiently than others [280]. Further examination of Duba null and phospho-mutant versions in vivo identified that Duba is essential for spermatogenesis [280]. In the late stages of spermatogenesis, spermatid individualization occurs which requires non-apoptotic functions of caspases [280]. Because level of ubiquitylated proteins were lower in Duba null mutant testes, it is possible that DUBA regulate function of caspases through removing their inhibitory ubiquitylation [280].

Another study in spermatid individualization showed that Cullin3 E3 ligase is required for caspase activation in this process [206]. Cullin3 forms a complex with Roc1b, a small RING domain protein and the Klhl10 BTB domain protein to mediate protein ubiquitylation at the onset of spermatid individualization [206].

In addition to Dronc, drICE and Dcp-1, the caspase-8 homolog DREDD was shown to be ubiquitylated. DREDD was shown to regulate innate immunity responses in flies [281]. It functions in the Immune Deficiency (IMD) pathway, which is similar to
mammalian NF-κB signaling. Upon microbial infection, DIAP2 was shown to bind DREDD at its Death Effector Domain 1 (DED1) and mediates its K63 poly-ubiquitylation [282]. This ubiquitylation leads to processing and activation of DREDD [282]. Activated DREDD cleaves IMD protein, which exposes an IBM that is required for its interaction with DIAP2 [196, 282]. DIAP2-mediated K63-poly-ubiquitylation of IMD protein provides a scaffolding complex with other components of IMD signaling [196]. Impaired ubiquitylation of DREDD and IMD proteins hinders IMD signaling pathway to respond to microbial infection [196, 282]. These studies elegantly suggest that caspases could be ubiquitylated for activation as well as inhibition.
CHAPTER II

An inhibitory mono-ubiquitylation of the Drosophila initiator caspase Dronc functions in both apoptotic and non-apoptotic pathways

Author Contributions

Andreas Bergmann and H.E. Kamber Kaya designed the experiments. H.E. Kamber Kaya performed the experiments. H.E. Kamber Kaya and Andreas Bergmann wrote the manuscript. H.E. Kamber Kaya, A. Bergmann, P. Meier and M. Ditzel provided feedback.

Summary

Apoptosis is an evolutionary conserved cell death mechanism, which requires activation of initiator and effector caspases. The Drosophila initiator caspase Dronc, the ortholog of mammalian Caspase-2 and Caspase-9, has an N-terminal CARD domain that recruits Dronc into the apoptosome for activation. In addition to its role in apoptosis, Dronc also has non-apoptotic functions such as compensatory proliferation. One mechanism to control the activation of Dronc is ubiquitylation. However, the mechanistic details of ubiquitylation of Dronc are less clear. For example, monomeric inactive Dronc is subject to non-degradative ubiquitylation in living cells, while ubiquitylation of active apoptosome-bound Dronc triggers its proteolytic degradation in apoptotic cells. Here, we examined the role of non-degradative ubiquitylation of Dronc in living cells in vivo, i.e.
in the context of a multi-cellular organism. Our \textit{in vivo} data suggest that in living cells Dronc is mono-ubiquitylated on Lys78 (K78) in its CARD domain. This ubiquitylation prevents activation of Dronc in the apoptosome and protects cells from apoptosis. Furthermore, K78 ubiquitylation plays an inhibitory role for non-apoptotic functions of Dronc. We provide evidence that not all of the non-apoptotic functions of Dronc require its catalytic activity. In conclusion, we demonstrate a mechanism whereby Dronc’s apoptotic and non-apoptotic activities can be kept silenced in a non-degradative manner through a single ubiquitylation event in living cells.

\textbf{Introduction}

In multicellular organisms, cells have a turning point in their lives to commit to either living or dying. Cells which are committed to die can employ different forms of cell death, the most common one being a conserved form of programmed cell death, called apoptosis \cite{2, 283}. Apoptosis plays important roles during development, to maintain tissue homeostasis in adult organisms and in response to stress conditions \cite{4, 284}. Studies aimed at the elucidation of regulatory pathways of apoptosis are of outstanding importance because dysregulation of apoptosis can lead to many disorders, including neurodegenerative diseases and cancer \cite{147, 285}. The fruit fly \textit{Drosophila melanogaster} provides an excellent model system in which to study the molecular mechanisms of apoptosis owing to its genetic conservation with mammals \cite{50}, low genetic redundancy of the apoptotic factors, and a variety of well-established genetic techniques that allow to easily manipulate gene function in specific tissue types and even individual cells.
Caspases, a highly conserved family of Cysteine (Cys) proteases, play a pivotal role in the regulation and execution of apoptosis. Caspases are produced as inactive monomeric zymogenes that consist of three domains, an N-terminal pro-domain, a large subunit containing the catalytic Cys residue, and a C-terminal small subunit. There are two types of apoptotic caspases: initiator caspases such as Caspase-2, Caspase-9 and the Drosophila ortholog Dronc; and effector caspases such as the Caspase-3, Caspase-7 and the Drosophila orthologs Drice and Dcp-1 [24, 286]. The prodomains of initiator caspases carry protein/protein interaction motifs such as the Caspase Recruitment Domain (CARD) [287]. The scaffolding protein Apaf-1 and its Drosophila ortholog Dark also carry an N-terminal CARD domain [71-73, 288]. In apoptotic cells, through CARD/CARD interactions with Dark, Dronc is recruited into and activated by a death-inducing protein complex, termed apoptosome [95, 289]. Effector caspases which have short prodomains without protein/protein interaction motifs, are activated by the apoptosome through proteolytic cleavages between their subunits.

Interestingly, correct stoichiometry between Dronc and Dark molecules is important for execution of apoptosis [99]. There is feedback inhibition between Dronc and Dark. Overexpression of one protein triggers degradation of the other one [99] ensuring that the levels of functional apoptosome units are low under these conditions. Only if both proteins are co-expressed can a significant apoptotic phenotype be recorded.

Inhibitor of Apoptosis Proteins (IAPs) restrict apoptosis by inhibiting caspases [290, 291]. IAPs are characterized by the presence of one to three Baculovirus IAP Repeats (BIR) and some bear a C-terminal RING domain that provides E3 ligase activity
for ubiquitylation [290, 292, 293]. In living cells, *Drosophila* IAP1 (Diap1) interacts with Dronc, Drice and Dcp-1 through the BIR domains [49]. Importantly, binding of Diap1 to caspases is not sufficient for their inhibition; ubiquitylation by the RING domain of Diap1 is required for full inhibition of these caspases [49, 78, 89]. In dying cells, the pro-apoptotic proteins Reaper (Rpr), Hid and Grim bind to Diap1 and change the E3 ligase activity of the RING domain which promotes auto-ubiquitylation and degradation of Diap1 [36-38, 82, 84, 85, 272, 273]. This leads to release of Dronc from Diap1 inhibition and free Dronc monomers can be recruited into the Dark apoptosome.

Ubiquitylation is a post-translational modification, which results from conjugation of a protein called Ubiquitin to lysine residues of substrates either as a single moiety (mono-ubiquitylation) or by conjugation of ubiquitin chains (poly-ubiquitylation) [210, 294]. The fate of a poly-ubiquitylated protein depends on the nature of the ubiquitin linkage. For example, K48 poly-ubiquitylation triggers proteolytic degradation of target proteins, while K63 poly-ubiquitylation regulates non-degradative events such as cell signaling [295-298]. In contrast, mono-ubiquitylation of a protein is usually not associated with protein degradation. Mono-ubiquitylation of target proteins is involved in DNA repair and endocytosis or may regulate translocation and interaction with other proteins [296, 297].

Both mammalian and *Drosophila* caspases are subject of regulatory ubiquitylation mediated by IAPs [242, 290, 292, 293, 299, 300]. For example, previous studies conducted *in vitro* and by transfection experiments in cell culture demonstrated that in *Drosophila* Dronc is ubiquitylated by Diap1 [78, 80, 89]. The importance of the RING
domain for control of Dronc activity became clear from genetic analysis. *diap1* mutants lacking the RING domain are embryonic lethal due to massive apoptosis [38]. Consistently, loss of the RING domain of Diap1 triggers processing and activation of Dronc [89] suggesting that ubiquitylation negatively regulates Dronc processing and activation. Initially, it was proposed that ubiquitylated Dronc is degraded by the proteasome [79, 80, 180]. However, we showed recently that the level of Dronc protein does not increase in proteasome mutants [301] suggesting that Dronc is not subject of proteasome-mediated degradation. In fact, the control of Dronc activity by ubiquitylation is much more complex than initially anticipated. In living cells, free monomeric Dronc is subject to non-degradative ubiquitylation, while processed and activated Dronc in the Dark apoptosome is degraded in a Diap1-dependent manner [89, 99]. That raises the question about the nature and function of non-degradative ubiquitylation of free monomeric Dronc in living cells.

Here, we report that in living cells Dronc is mono-ubiquitylated at Lysine 78 (K78) in its CARD domain. To examine the role of K78 mono-ubiquitylation of Dronc, we mutated this residue to non-ubiquitable Arginine (K78R). Dronc$^{\text{K78R}}$ and Dronc$^{\text{wt}}$ display similar enzymatic activities *in vitro*. However, Dronc$^{\text{K78R}}$ is easier incorporated into the Dark apoptosome, is more efficiently processed and thus has higher enzymatic activity there. These data suggest that K78 ubiquitylation inhibits incorporation of Dronc into the Dark apoptosome. Surprisingly, Dronc$^{\text{K78R}}$ also suppresses some of the phenotypes associated with catalytic inactivity of Dronc such as lethality, loss of compensatory proliferation and defects in male genitalia rotation. These observations
provide evidence that K78 mono-ubiquitylation also controls non-apoptotic functions of Dronc and suggest that not all of the non-apoptotic functions of Dronc require its catalytic activity. In summary, this in vivo study provides a mechanistic link of how ubiquitylation of an initiator caspase can control its activity in both apoptotic and non-apoptotic pathways in a non-degradative manner.

Results

Dronc is mono-ubiquitylated in living cells

Because available anti-Dronc antibodies perform poorly in immunoprecipitation (IP) experiments, we took advantage of the Gal4/UAS system [302] and expressed Flag-tagged Dronc (Flag-Dronc) [98] ubiquitously using the daughterless-Gal4 (da-Gal4) driver (denoted da>Flag-Dronc). Expression of da>Flag-Dronc in whole animals does not cause any significant developmental, apoptotic or lethality phenotypes. To examine the functionality of Flag-Dronc, we tested if it can rescue the lethal phenotype of strong dronc mutants (droncI24/droncI29) [61]. We indeed observed that da>Flag-Dronc is able to rescue the pupal lethality caused by dronc null mutations and can be activated in the apoptosome (Figure 2.1A, B).

To address the status of Dronc ubiquitylation, we immunoprecipitated Flag-Dronc from embryonic, larval, pupal and adult fly extracts and blotted with FK1 and FK2 antibodies that bind to ubiquitin-conjugated proteins, but not free, unconjugated ubiquitin. FK2 antibody binds to mono- and poly-ubiquitylated proteins, while FK1 antibody detects only poly-ubiquitin-conjugated proteins [303]. Blotting the IPs with FK2 antibody revealed high molecular poly-ubiquitin species; however, these are comparable
to the control IPs and may represent unspecific co-immunoprecipitated proteins (Figure 2.2A). In contrast, in the 60 kDa range, FK2 detected a single band specifically in Dronc IPs (Figure 2.2A, arrow). This band is found in all developmental stages tested from embryos to adults. The FK1 antibody did not detect this band (Figure 2.2A). Flag-Dronc has an estimated molecular weight (MW) of 51 kDa, and adding one ubiquitin moiety of ~8.5 kDa results in a combined MW of about 60 kDa, suggesting that this band may correspond to mono-ubiquitylated Flag-Dronc.

To further verify mono-ubiquitylation of Dronc in vivo, we co-expressed da>Flag-Dronc and 6xHis-tagged ubiquitin (6xHis-ubiquitin) and pulled down all ubiquitylated proteins using Ni-NTA agarose beads. Blotting for Flag-Dronc revealed a single band of about 60kDa, that was not present in the control IP in which we only expressed 6xHis-ubiquitin (Figure 2.2B). This result further confirms that Dronc is ubiquitylated in vivo and the differential detection by FK2, but not FK1, suggests that it is – surprisingly - mono-ubiquitylated.

As further evidence that this modification of Flag-Dronc corresponds to ubiquitylation, we incubated larval Flag-Dronc immunoprecipitates with a de-ubiquitylating enzyme, USP2, which removes conjugated ubiquitin from target proteins. Consistently, in immunoblots, the FK2 signal is strongly reduced after USP2 incubation compared to the control (Figure 2.2C, upper panel, arrow; quantified in 2.2C’). Interestingly, although the majority of Flag-Dronc is de-ubiquitylated after USP2 incubation, this does not result in a significant reduction of the molecular weight (MW)
Figure 2.1. *Flag-Dronc*<sup>wt</sup> is functional.

(A) *Flag-Dronc* can rescue the lethality associated with *dronc* null mutations.

(B) *Flag-Dronc* can be activated in the apoptosome. Expression of either *da>*Flag-Dronc or *GMR-Dark* does not lead to any caspase (cleaved caspase-3, cc3) activity. However, when these transgenes are co-expressed (*da>*Flag-Dronc+*GMR-Dark*), caspase activity is increased in the posterior domain.
Figure 2.2. Dronc is mono-ubiquitylated at K78 in living cells for its inhibition.

Arrows indicate mono-ubiquitylated Dronc. Asterisks denote unspecific bands.

(A) Immunoprecipitates with Flag antibody from *da>Flag-Dronc* extracts of the indicated developmental stages were examined for Flag-Dronc ubiquitylation with FK2 and FK1 antibodies.

(B) Extracts from *da>Flag-Dronc+6xHis-ubiquitin* larvae were used to pull down 6xHis-tagged ubiquitylated proteins. Flag antibody was used to detect Flag-Dronc.

(C, C’) USP2 de-ubiquitinase can remove the conjugated mono-ubiquitin on Flag-Dronc. (C’) is the quantification of the Flag-Dronc bands, n=3. The FK2 signal in (C’) is normalized against immunoprecipitated Flag-Dronc. The removal of mono-ubiquitin does not cause a significant change in MW of Flag-Dronc.

(D, D’) Heterozygous *diap1<sup>2</sup>* mutants display reduced Flag-Dronc mono-ubiquitylation (quantified in D’, n=2). The loss of mono-ubiquitin does not significantly change the MW of Flag-Dronc.

(E) Domain structure of Dronc, showing relative position of K78 in the CARD domain. L = large subunit; S = small subunit.

(F, F’) Flag-Dronc<sup>K78R</sup> mono-ubiquitylation is significantly reduced compared to Flag-Dronc<sup>wt</sup>. (quantified in F’, n=5). The loss of mono-ubiquitin does not significantly change the MW of Flag-Dronc<sup>K78R</sup>.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05; ** P<0.01.
of non-ubiquitylated Flag-Dronc (Figure 2.2C, lower panel). Nevertheless, this characterization indicates that Flag-Dronc is mono-ubiquitylated under in vivo conditions.

We were also interested to identify the ubiquitin ligase that mediates mono-ubiquitylation of Dronc. One good candidate is Diap1 which has been shown to ubiquitylate Dronc in vitro [78, 80, 89]. Ideally, to test if Diap1 ubiquitylates Flag-Dronc in vivo, one should examine homozygous mutant diap1 animals for loss of ubiquitylation of Dronc. However, these animals are early embryonic lethal due to strong apoptosis induction by loss of Diap1 [38] which makes this analysis very difficult. Therefore, we examined Flag-Dronc immunoprecipitates from larvae that were heterozygous for the strong diap15 allele [36, 37]. Immunoprecipitates of Flag-Dronc from heterozygous diap15 extracts display a significant reduction of FK2 immunoreactivity (Figure 2.2D, upper panel; quantified in 7D’) suggesting that Diap1 is involved in mono-ubiquitylation of Flag-Dronc. However, as already noted above in the context of the USP2 experiments, the Flag immunoblots do not display a significant size difference between ubiquitylated and non-ubiquitylated Flag-Dronc (Figure 2.2D, lower panel). The reason for this unusual behavior is not known.

**Flag-Dronc is ubiquitylated at K78 in the CARD domain**

To identify the ubiquitylated Lysine (K) residue, we submitted the 60kDa band from immunoprecipitated Flag-Dronc samples from both larval and pupal stages to mass-spectrometry (LC-MS/MS) analysis. Both analyses showed that Flag-Dronc is ubiquitylated at K78 (Figure 2.3A). To also examine for poly-ubiquitylation, we submitted higher molecular weight bands of the Flag immunoprecipitates for LC-MS/MS
analysis. However, there was no trace of ubiquitylation. In addition to mono-ubiquitylation of K78, we also observed phosphorylation of Ser130, an inhibitory modification of Dronc that has previously been reported [98]. Confirmation of a known modification of Dronc validates the LC-MS/MS approach. Importantly, LC-MS/MS analysis of apoptotic extracts (induced by hs-hid) revealed that the mono-ubiquitylation at K78 is absent (Figure 2.3B). This observation suggests that K78 mono-ubiquitylation is a feature of Dronc in living cells and that it may control (inhibit) the apoptotic activity of Dronc.

To determine whether DIAP1 can ubiquitylate Dronc at K78, we performed in vitro ubiquitylation assays of Dronc with Diap1 as E3 ubiquitin ligase and analyzed in vitro ubiquitylated Dronc by mass spectrometry. As E2 conjugating enzymes we used either human UBE2D2 or Drosophila UBCD1. In both cases, Dronc was found to be ubiquitylated at K78 by DIAP1 in vitro (Figure 2.3C, D), suggesting that DIAP1 can mediate K78 ubiquitylation of Dronc.

K78 resides in the CARD domain of Dronc (Figure 2.2E) which interacts with the CARD domain of Dark for recruitment of Dronc into the apoptosome. To study the role of K78 ubiquitylation, we mutated K78 to Arginine (R) and generated transgenic UAS-Flag-Dronc$^{K78R}$ flies by phiC31-based site-specific integration [304, 305]. In addition, we combined the K78R mutation with a mutation that changes the catalytic Cys (C) to Ala (A) (C318A), generating transgenic UAS-Flag-Dronc$^{K78RC318A}$ flies. As controls, we generated UAS-Flag-Dronc$^{wt}$, a catalytically inactive Dronc (UAS-Flag-Dronc$^{C318A}$) and
**A.** Surviving conditions, *in vivo*

**B.** Apoptotic conditions, *in vivo*

**C.** *In vitro* ubiquitylation assay with human UBE2D2 and DIAP1

**D.** *In vitro* ubiquitylation assay with Drosophila UBCD1 and DIAP1
Figure 2.3. LC-MS/MS analysis shows that Dronc is ubiquitylated at K78.

(A, B) Of the peptides obtained by Chymotrypsin digests of immunoprecipitated Dronc from larval and pupal extracts under surviving conditions (A), only the peptide K78ITQRGPT was found to carry the di-Glycine signature indicative of ubiquitin modification. di-Glycine adds 114 Da to this peptide. Correspondingly, all b peaks of this peptide obtained under surviving conditions (A) are shifted compared to the b peaks under apoptotic conditions (B); see asterisk at peak b1 as example.

(C, D) Mass-spec analyses of in vitro ubiquitylated Dronc with Diap1 as E3 ligase and either human UBE2D2 (C) or Drosophila UBCD1 (D) as E2 conjugating enzymes show that K78 can be ubiquitylated by DIAP1. Arrows indicate 114 Da mass shift due to ubiquitylation on K78.
empty vector transgenic flies. Because all constructs are inserted in the same landing site in the genome (VK37 on 2nd chromosome), the expression and protein levels of these Dronc constructs are expected to be the same which was confirmed by immunoblotting (Figure 2.9H). To test whether da>Flag-Dronc\textsuperscript{K78R} mutant flies lose the mono-ubiquitylation signal, we immunoprecipitated Dronc from larval samples and probed immunoblots with FK2 antibody. da>Flag-Dronc\textsuperscript{K78R} larval samples showed significantly reduced levels of mono-ubiquitylation (Figure 2.2F, arrow; quantified in Figure 2.2F’), suggesting that Flag-Dronc\textsuperscript{K78R} is less efficiently ubiquitylated compared to Flag-Dronc\textsuperscript{wt}. However, because K78 is the only Lys residue being detected by LC-MS/MS, we expected a complete loss of ubiquitylation in the Flag-Dronc\textsuperscript{K78R} mutant. Although significantly reduced, the mono-ubiquitylation signal is not completely lost (Figure 2.2F’) suggesting that in the absence of K78 as major ubiquitin acceptor, another Lys residue may be used as alternative ubiquitylation site (see Discussion). Nevertheless, the K78R mutation revealed that K78 of Dronc is a major ubiquitin acceptor. Interestingly also, as already observed in the USP2 and diap1\textsuperscript{5} experiments, the MW of ubiquitylated and non-ubiquitylated Dronc is not significantly different (Figure 2.2F, lower panel).

\textit{Flag-Dronc}\textsuperscript{K78R} shows enhanced genetic interaction with Dark in a Diap1-dependent manner

Formation of the apoptosome is essential for activation of Dronc. Interestingly, a recent structural report about the \textit{Drosophila} apoptosome revealed that K78 forms an intramolecular hydrogen bond with a critical residue (Q81) that is required for interaction of the CARD domains of Dronc and Dark for apoptosome formation [95] (Figure 2.4).
Figure 2.4. Atomic interactions between K78 and Gln81 (Adapted from [95]).

(A) Under apoptotic conditions, K78 at the CARD of Dronc forms an intramolecular hydrogen bond with Gln81 that is an essential residue for interactions between CARDs of Dark and Dronc. Intramolecular interactions between K78 and Gln81 were suggested to support interactions of Gln81 with CARD of Dark.

(B) Under surviving conditions, K78 is ubiquitylated, it cannot form a hydrogen bond with Gln81, which may lead to a weaker interaction between Gln81 and corresponding residue in CARD of Dark. This may hinder apoptosome formation.
Therefore, we hypothesized that mono-ubiquitylation of Dronc at K78 inhibits the interaction with the CARD of Dark, effectively blocking recruitment of Dronc into the apoptosome under surviving conditions (Figure 2.4). To test this hypothesis in vivo, we used genetic and biochemical approaches.

In genetic experiments, we tested whether apoptosis is induced when the K78 mono-ubiquitylation is lost in animals expressing da>Flag-Dronc\(^{K78R}\). However, similar to da>Flag-Dronc\(^{wt}\), expression of da>Flag-Dronc\(^{K78R}\) does not induce a significant apoptotic phenotype or even cause lethality. This is most likely due to the feedback inhibition mechanism between Dronc and Dark according to which overexpressed Dronc destabilizes Dark [99], keeping the number of active apoptosome units low (see Discussion).

Nevertheless, combined expression of Flag-Dronc\(^{wt}\) and Dark (tagged with GFP (GFP-Dark) [99]) with GMR-GAL4 in the posterior eye imaginal disc induces apoptosis, causing eyes of reduced size with pigment loss (Figure 2.5A) and enhanced pupal lethality. Therefore, we asked whether loss of K78 mono-ubiquitylation causes increased activity of Flag-Dronc\(^{K78R}\) in the presence of mis-expressed GFP-Dark [99]. Indeed, we found that the adult eyes of GMR>Flag-Dronc\(^{K78R}\)+GFP-Dark flies are significantly smaller than GMR>Flag-Dronc\(^{wt}\)+GFP-Dark eyes (Figure 2.5A, 2B). In addition, the pupal lethality was significantly increased in GMR>Flag-Dronc\(^{K78R}\)+GFP-Dark compared to GMR>Flag-Dronc\(^{wt}\)+GFP-Dark (Figure 2.5C).
Figure 2.5. Loss of K78 ubiquitylation results in increased Dronc activity in the apoptosome.

(A-C) Flag-Dronc<sup>K78R</sup> and GFP-Dark co-expression under GMR-Gal4 control results in significantly smaller eyes and a lower survival rate than GMR>Flag-Dronc<sup>wt</sup>+GFP-Dark. Control flies just expressing GMR>Flag-Dronc<sup>wt</sup>, GMR>Flag-Dronc<sup>K78R</sup> or GMR>GFP-Dark alone, show wild type eye phenotype. (B) Quantification of the eye sizes in (A). n=19 for GMR>Flag-Dronc<sup>wt</sup>+GFP-Dark; n= 20 for GMR>Flag-Dronc<sup>K78R</sup>+GFP-Dark. (C) Quantification of the reduced survival of GMR>Flag-Dronc<sup>K78R</sup>+GFP-Dark compared to GMR>Flag-Dronc<sup>wt</sup>+GFP-Dark.

(D,E) Significantly higher TUNEL labeling in the GMR-expression domain (arrows) of GMR>Flag-Dronc<sup>K78R</sup>+GFP-Dark compared to GMR>Flag-Dronc<sup>wt</sup>+GFP-Dark eye.
imaginal discs of 3\textsuperscript{rd} instar larvae. GFP labels Dark. (E) Quantification of TUNEL positive cells in (D). n=7 for both genotypes.

(F) \textit{In vitro} caspase activity assays of adult fly head extracts show significantly higher caspase activity with $GMR>\text{Flag-Dronc}^{K78R} + \text{GFP-Dark}$ towards Ac-DEVD-AMC substrate than $GMR>\text{Flag-Dronc}^{wt} + \text{GFP-Dark}$.

For quantifications, the student’s t-test was used. Error bars are SD. ** P<0.01; *** P<0.001; **** P<0.0001.
To understand whether this phenotype is due to increased apoptotic activity of Flag-Dronc\textsuperscript{K78R}, we examined 3\textsuperscript{rd} instar larval eye discs for apoptosis using TUNEL labeling. Parallel to the adult eye phenotypes, we observed significantly more apoptosis in the GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark eye imaginal discs (Figure 2.5D, E). In addition, fluorimetric caspase activity assays with extracts from GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark heads showed a significantly higher cleavage activity towards the synthetic DEVD substrate than GMR>Flag-Dronc\textsuperscript{wt}+GFP-Dark (Figure 2.5F). These data suggest that loss of K78 mono-ubiquitylation increases the apoptotic activity of Dronc\textsuperscript{K78R} in the Dark apoptosome.

To examine the role of Diap1 for K78 mono-ubiquitylation of Flag-Dronc, we compared the eye phenotypes of GMR>Flag-Dronc\textsuperscript{wt}+GFP-Dark and GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark in a heterozygous diap1\textsuperscript{5} background. diap1 heterozygosity strongly enhanced the eye phenotype and lethality of GMR>Flag-Dronc\textsuperscript{wt}+GFP-Dark animals (Figure 2.6A-C). However, loss of one copy of diap1 only weakly enhances the eye phenotype and lethality of GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark animals (Figure 2.6A-C). These genetic interaction data suggest that K78 ubiquitylation depends on Diap1.

Dark has a C-terminal caspase cleavage site that is thought to destabilize Dark, thus reducing its apoptosis-promoting activity [99, 100]. Consistently, a cleavage resistant version of Dark (Dark\textsuperscript{V}) showed a hypermorphic phenotype [100]. Therefore, in theory, Dark\textsuperscript{V} should uncouple the anti-apoptotic feedback of Dronc on Dark. However, experimentally, that was not observed [99]. Co-expression of GMR>Dronc\textsuperscript{wt}+GFP-Dark\textsuperscript{V} caused a similar small eye phenotype compared to GMR>Dronc\textsuperscript{wt}+GFP-Dark\textsuperscript{wt} [99].
Figure 2.6. Heterozygous diap1 melts mutant strongly enhances GMR>FlagDrone\textsuperscript{wt} +GFP-Dark eye phenotype, but only weakly enhances GMR>Flag-Drone\textsuperscript{K78R}+GFP-Dark.

(A–C) Loss of one copy of diap1 strongly enhances eye phenotype of GMR>Flag-Drone\textsuperscript{wt}+GFP-Dark (quantified in B) and causes a significant increase in lethality (quantified in C). In contrast, diap1 heterozygosity only weakly enhances GMR>Flag-Drone\textsuperscript{K78R}+GFP-Dark eye phenotype (quantified in B) and lethality (quantified in C).

(B) Quantification of eye size phenotypes in (A). n=9 for GMR>Flag-Drone\textsuperscript{wt}+GFP-Dark, n=11 for GMR>Flag-Drone\textsuperscript{wt}+GFP-Dark+diap1\textsuperscript{5/+}, n=8 for GMR>Flag-Drone\textsuperscript{K78R}+GFP-Dark, n=11 for GMR>Flag-Drone\textsuperscript{K78R}+GFP-Dark+diap1\textsuperscript{5/+}

(C) Quantification of eclosion rates of GMR>Flag-Drone\textsuperscript{wt}+GFP-Dark and GMR>Flag-Drone\textsuperscript{K78R}+GFP-Dark with or without loss of one copy of diap1.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05; *** P<0.001; ns – not significant.
Figure 2.7. Cleavage resistant Dark\textsuperscript{V}, but not cleaved-mimic Dark\textsuperscript{CC}, can form a more functional apoptosome with Flag-Dronc\textsuperscript{K78R} than with Flag-Dronc\textsuperscript{wt}.

(A) Expression of GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark resulted in significantly smaller eyes than GMR>Flag-Dronc\textsuperscript{wt}+GFP-Dark. Expression of GMR>GFP-Dark\textsuperscript{V} alone does not have any eye phenotype.

(B) Quantification of eye size phenotypes in (A). n=10 for each genotype

(C) Eclosion rates of flies expressing GMR>Flag-Dronc\textsuperscript{K78R}+GFP-Dark\textsuperscript{V} are significantly smaller than GMR>Flag-Dronc\textsuperscript{wt}+GFP-Dark\textsuperscript{V}.
(D, E) Expression of $Dark^{CC}$ and $Flag-Dronc^{wt}$ or $Flag-Dronc^{K78R}$ under $GMR-Gal4$ control does not lead to any eye phenotype or lethality.

For quantifications, the student’s t-test was used. Error bars are SD. ** P<0.01.
Thus, although Dark$^V$ was suggested to be more active than Dark$^w^t$, expression of either transgene with Drone$^w^t$ did not change the equilibrium of the apoptosome activation [99]. Therefore, we examined whether co-expression of Flag-Dronc$^{K78R}$ with GFP-Dark$^V$ under GMR control is sufficient to shift the equilibrium of apoptosome formation towards higher induction of apoptosis. Indeed, GMR>Flag-Dronc$^{K78R}$+GFP-Dark$^V$ executed more apoptosis compared to GMR>Flag-Dronc$^w^t$+GFP-Dark$^V$ (Figure 2.7A). Both the adult eye phenotype and the pupal lethality are worsened significantly in GMR>Dronc$^{K78R}$+GFP-Dark$^V$ flies (Figure 2.7A-C).

On the other hand, a mutant that mimics the cleavage product of Dark (Dark$^{CC}$) is unstable and failed to induce apoptosis when co-expressed with Drone$^w^t$ [99]. Consistently, co-expression of GFP-Dark$^{CC}$ did not display any abnormal phenotype with either Flag-Dronc$^w^t$ or Flag-Dronc$^{K78R}$ (Figure 2.7D, E). These findings are consistent with the notion that Flag-Dronc$^{K78R}$ requires functional Dark for increased activity.

The $K78R$ mutation increases processing of Dronc through enhanced interaction with Dark

To examine if the increased caspase activity of Flag-Dronc$^{K78R}$ is due to increased intrinsic catalytic activity, we performed in vitro cleavage assays with bacterially expressed 6xHis-Dronc$^w^t$, 6xHis-Dronc$^{K78R}$, 6xHis-Dronc$^{C318A}$ and 6xHis-Dronc$^{K78RC318A}$. Because bacteria lack an ubiquitin system, 6xHis-Dronc$^w^t$ is not modified by ubiquitin enabling us to directly compare the intrinsic activities of the Dronc variants. In these experiments, we first tested the ability of the Dronc constructs to auto-process [96, 97]. Both 6xHis-Dronc$^w^t$ and 6xHis-Dronc$^{K78R}$ proteins are able to auto-process to a similar
extend (Figure 2.8A). In contrast, the catalytic mutant Dronc\textsuperscript{C318A} and double mutant Dronc\textsuperscript{K78RC318A} fail to auto-process (Figure 2.8A), consistent with the expectation.

Next, we performed \textit{in vitro} cleavage assays of these Dronc preparations with its known cleavage target drICE [96, 97] which is Myc-tagged and carries a mutation in the catalytic site (Myc-drICE\textsuperscript{C211A}) to block auto-processing of drICE. While the catalytic mutants 6xHis-Dronc\textsuperscript{C318A} and 6xHis-Dronc\textsuperscript{K78RC318A} failed to cleave Myc-drICE\textsuperscript{C211A}, both 6xHis-Dronc\textsuperscript{wt} and 6xHis-Dronc\textsuperscript{K78R} processed Myc-drICE\textsuperscript{C211A} \textit{in vitro} (Figure 2.8B). However, the cleavage activities of 6xHis-Dronc\textsuperscript{wt} and 6xHis-Dronc\textsuperscript{K78R} are very similar in these assays suggesting that there are no intrinsic differences in the catalytic activities of 6xHis-Dronc\textsuperscript{wt} and 6xHis-Dronc\textsuperscript{K78R}. Furthermore, these data imply that the K78R mutation does not cause any structural defect to Dronc\textsuperscript{K78R}. However, \textit{in vivo}, in the presence of Dark, Flag-Dronc\textsuperscript{K78R} has a higher catalytic activity than Flag-Dronc\textsuperscript{wt} (Figure 2.5) suggesting that Dronc\textsuperscript{K78R} requires Dark for increased catalytic activity.

Consistent with the increased catalytic activity of Flag-Dronc\textsuperscript{K78R} in the presence of Dark, a significantly higher amount of Flag-Dronc\textsuperscript{K78R} is found in the processed form compared to Flag-Dronc\textsuperscript{wt} in immunoblots of total extracts from \textit{da}>Flag-Dronc\textsuperscript{wt} + GFP-Dark and \textit{da}>Flag-Dronc\textsuperscript{K78R} + GFP-Dark larvae (Figure 2.8C, C'). To understand the mechanism of increased processing and catalytic activity of Flag-Dronc\textsuperscript{K78R} in the Dark apoptosome, we examined the interaction between Dronc\textsuperscript{K78R} and Dark. Because specific antibodies to Dark do not exist, we used the GFP-Dark transgenes [99] to immunoprecipitate GFP-Dark and associated Flag-Dronc. To avoid embryonic lethality of \textit{da}>Flag-Dronc\textsuperscript{K78R}+GFP-Dark, \textit{Gal80}ts was used to control the expression of \textit{UAS-
**GFP-Dark** and **UAS-Flag-Dronc** transgenes. Using **Gal80**

\( \text{da} \text{>Flag-Dronc}^{\text{wt}} \text{+GFP-Dark} \), \( \text{da} \text{>Flag-Dronc}^{\text{K78R}} \text{+GFP-Dark} \) and **EV** (empty vector)\(+GFP-Dark\) as control were induced for 24 h at 29°C and larval extracts were analyzed for Flag-Dronc and GFP-Dark. Longer induction periods (e.g. ≥48 h) also caused lethality. Consistent with a previous report [99], compared to the **EV** control, expression of \( \text{da} \text{>Flag-Dronc}^{\text{wt}} \text{+GFP-Dark} \) and \( \text{da} \text{>Flag-Dronc}^{\text{K78R}} \text{+GFP-Dark} \) reduces Dark’s protein stability, as shown for GFP-Dark in Figure 2.8D (top panel). In co-IP experiments, we detect an increased interaction between Flag-Dronc\(^{\text{K78R}}\) and GFP-Dark compared to Flag-Dronc\(^{\text{wt}}\) and GFP-Dark (Figure 2.8D, bottom panel). In addition, the ratio between processed versus unprocessed Dronc is significantly increased for Flag-Dronc\(^{\text{K78R}}\) in complex with GFP-Dark compared to Flag-Dronc\(^{\text{wt}}\) (Figure 2.8D, bottom panel; quantified in 2.7D’), consistent with the increased apoptosis in imaginal discs and head extracts (Figure 2.5). These results suggest that compared to Flag-Dronc\(^{\text{wt}}\), Flag-Dronc\(^{\text{K78R}}\) interacts stronger with Dark and is more efficiently processed for apoptosis induction.

Taken together, these data suggest that living cells are protected from apoptosis by keeping Dronc at least partially inactive through K78 mono-ubiquitylation which appears to block recruitment into the Dark apoptosome. However, when cells are undergoing apoptosis, K78 mono-ubiquitylation is no longer present, allowing Dronc to interact with Dark in the apoptosome and induce cell death.

**K78R is an intragenic suppressor of the lethality associated with loss of catalytic activity of Dronc**
Figure 2.8. Biochemical characterization of Flag-Dronc\textsuperscript{K78R}.

(A) Bacterially expressed 6xHis-Dronc\textsuperscript{wt} and 6xHis-Dronc\textsuperscript{K78R} constructs display similar auto-processing activities. 6xHis-Dronc\textsuperscript{C318A} and 6xHis-Dronc\textsuperscript{K78RC318A} do not show any auto-processing.

(B) \textit{In vitro} caspase cleavage assays show that 6xHis-Dronc\textsuperscript{wt} and 6xHis-Dronc\textsuperscript{K78R} cleave Myc-Drice\textsuperscript{C211A} with similar activities. 6xHis-Dronc\textsuperscript{C318A} and 6xHis-Dronc\textsuperscript{K78RC318A} cannot cleave Myc-Drice\textsuperscript{C211A}.

(C, C') 3\textsuperscript{rd} instar lysates of da\textgreater GFP-Dark\textgreater Flag-Dronc\textsuperscript{wt} and da\textgreater GFP-Dark\textgreater Flag-Dronc\textsuperscript{K78R} show that in the presence of Dark, Flag-Dronc\textsuperscript{K78R} is processed significantly more than Flag-Dronc\textsuperscript{wt}. In (C'), the average of 4 immunoblots is plotted.

(D) GFP-Dark interacts with Flag-Dronc\textsuperscript{wt} and Flag-Dronc\textsuperscript{K78R}. GFP-immunoprecipitates of 3\textsuperscript{rd} instar larval extracts from da\textgreater GFP-Dark\textgreater Flag-Dronc\textsuperscript{wt}, da\textgreater GFP-Dark\textgreater Flag-Dronc\textsuperscript{K78R} and da\textgreater GFP-Dark\textgreater EV (Flag-Empty Vector) animals, probed with anti-GFP antibody (upper panel) and anti-Flag antibody (lower panel). There is a stronger interaction between GFP-Dark and Flag-Dronc\textsuperscript{K78R}, resulting in significantly more efficient procession of Flag-Dronc\textsuperscript{K78R} compared to Flag-Dronc\textsuperscript{wt}.

(D') Relative ratio of processed and unprocessed Flag-Dronc proteins in the Dark apoptosome. Flag-Dronc\textsuperscript{K78R} is more efficiently processed than Flag-Dronc\textsuperscript{wt}. The average of 3 immunoblots is plotted.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05
Next, we examined the physiological role of K78 mono-ubiquitylation of Dronc. For this, we expressed wild-type and mutant Flag-Dronc transgenes using da-Gal4 in a dronc null background and scored for rescue. The null mutants used, dronc^{I24} and dronc^{I29}, have early stop codons at positions 28 and 53, respectively [61] and do not produce any Dronc protein. dronc^{I24}/dronc^{I29} null mutants display a strong semi-lethal phenotype. Less than 10% of the expected dronc homozygous mutant animals survive development (Figure 2.9A) and hatch as adults with wing abnormalities (Figure 2.9)[61].

Expression of da>Flag-Dronc^{wt} rescues the lethality of dronc null mutant flies, but it is only a partial rescue. There is still about a 35% lethality (Figure 2.9A), suggesting that da>Flag-Dronc^{wt} does not reach sufficient Dronc activity for full rescue. Interestingly, however, da>Flag-Dronc^{K78R} rescued the lethality of dronc null mutant significantly better than da>Flag-Dronc^{wt}. More than 80% of the expected progeny emerges as adults in the presence of Flag-Dronc^{K78R} (Figure 2.9A). Because these transgenes were obtained by phiC31 integration in the same landing site, the expression levels of all Flag-Dronc constructs are comparable (Figure 2.9H) and are not responsible for the observed differences. Therefore, this result further supports the notion that Flag-Dronc^{K78R} has more activity than Flag-Dronc^{wt} and thus can better substitute for the loss of endogenous dronc.

As expected, expression of catalytically inactive da>Flag-Dronc^{C318A} failed to rescue the lethality of dronc null mutants (Figure 2.9A). Surprisingly, however, expression of da>Flag-Dronc^{K78RC318A} which lacks the K78 mono-ubiquitylation site and is catalytically inactive (Figure 2.9A, B), did rescue the lethality of dronc null mutants to
Figure 2.9. Examination of K78 mono-ubiquitylation with respect to Dronc’s catalytic activity.

(A) da>Flag-Dronc\textsuperscript{wt}, da>Flag-Dronc\textsuperscript{K78R} and da>Flag-Dronc\textsuperscript{K78RC318A} can rescue the lethality of dronc\textsuperscript{I29} null mutants, whereas da>Flag-Dronc\textsuperscript{C318A} cannot.

(B) Quantification of the number of additional interommatidial cells (IOC) shown in (C-G). Genotypes are indicated. MARCM was used to express transgenic Flag-Dronc constructs in dronc\textsuperscript{I29} mutant cell clones. n= 10 for dronc\textsuperscript{I29} MARCM clones, n=11 for Flag-Dronc\textsuperscript{wt} in dronc\textsuperscript{I29} clones, n=7 for Flag-Dronc\textsuperscript{K78R} in dronc\textsuperscript{I29} clones, n=11 for Flag-Dronc\textsuperscript{K78RC318A} in dronc\textsuperscript{I29} clones, n=8 for Flag-Dronc\textsuperscript{C318A} in dronc\textsuperscript{I29} clones. Each n corresponds to an average of extra IOC of 3 clones. ns – not significant.
(C-G) Pupal retinae 48h after puparium formation expressing the indicated Flag-Dronc constructs in dronc129 MARCM clones. Clones are marked by GFP and are enclosed by white dashes in the right panels. Examples of extra IOC are marked with yellow arrows. Flag-Droncwt and Flag-DroncK78R rescue the IOC phenotype of dronc null mutants. However, Flag-DroncK78RC318A and Flag-DroncC318A fail to rescue this phenotype. Quantified in (B).

(H) Immunoblotting of lysates of each Flag-Dronc construct in the dronc124/dronc129 background shows similar expression levels.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001. ns – not significant.
Figure 2.10. Both Flag-Dronc$^{K78RC318A}$ and Flag-Dronc$^{C318A}$ cannot rescue the wing phenotype of dronc null mutants.

Compared to control flies (A, $w^{1118}$), wings from dronc null mutants are held-out, often irregularly shaped and less transparent (B). Often one wing is missing (see (F)). da$>$Flag-Dronc$^{K78RC318A}$ (E) and da$>$Flag-Dronc$^{C318A}$ (F) do not rescue this phenotype. In contrast, Flag-Dronc$^{wt}$ and Flag-Dronc$^{K78R}$ rescue the wing phenotype of dronc null mutants (C,D). However, these wings do not appear normal because of ectopic apoptosis (for details see reference [98]).
a significant degree! About 60% of dronc mutant flies survived when expressing \textit{da>Flag-Dronc^{K78RC318A}} compared to only 10% of dronc mutant flies expressing \textit{da>Flag-Dronc^{C318A}} (Figure 2.9A). Thus, the K78R mutation behaves as an intragenic suppressor of the lethality associated with loss of catalytic activity of Dronc. This result suggests that loss of K78 ubiquitylation can be advantageous for the survival of dronc mutant flies and can even –at least partially- overcome loss of the catalytic activity of Dronc.

\textit{Flag-Dronc^{K78RC318A} does not rescue the apoptotic phenotype of dronc null mutants}

Because of the intragenic suppression of the lethality of the catalytic \textit{dronc^{C318A}} mutant by the K78R mutation, we considered – although did not expect - that the K78R mutation would rescue the catalytic activity of Dronc\textit{^{C318A}} and thus the apoptotic phenotype of \textit{dronc} mutants. To test this possibility, we employed the developing \textit{Drosophila} retina which consists of individual units called ommatidia. In developing \textit{Drosophila} retinae, cells produced in excess between ommatidia (interommatidial cells, IOCs) are eliminated by apoptosis around 28-30h after puparium formation (APF) [17-20]. The retinal lattice is fully differentiated at 42-45h APF. Previous studies showed that \textit{dronc^{I24}} and \textit{dronc^{I29}} mutants fail to remove excess IOCs during development; about six additional IOCs remain per ommatidium in \textit{dronc} mutants (Figure 2.9B, C) [61, 105]. To understand the relationship between K78 mono-ubiquitylation and catalytic inactivity during developmental apoptosis, we generated \textit{dronc^{I29}} mutant clones expressing \textit{Flag-Dronc^{wt}}, \textit{Flag-Dronc^{K78R}}, \textit{Flag-Dronc^{C318A}} and \textit{Flag-Dronc^{K78RC318A}} by MARCM and examined the ability of these constructs to restore IOC apoptosis in the pupal retina of
dronc mosaics. As expected, while expression of Flag-Dronc\textsuperscript{wt} and Flag-Dronc\textsuperscript{K78R} rescues IOC apoptosis in dronc\textsuperscript{I29} mutant clones, Flag-Dronc\textsuperscript{C318A} does not (Figure 2.9D, E, G; quantified in Figure 2.9B). Importantly, although expression of Flag-Dronc\textsuperscript{K78RC318A} rescued the lethality of dronc mutant flies (Figure 2.9A), it does not restore IOC apoptosis in dronc mutant clones (Figure 2.9B, F). Consistently, da>Flag-Dronc\textsuperscript{K78RC318A} expression in dronc null background does not rescue the wing phenotype of dronc mutants (Figure 2.10E). In addition, Flag-Dronc\textsuperscript{K78RC318A} does not have catalytic activity \textit{in vitro} (Figure 2.8A, B).

Therefore, as expected, these findings suggest that the K78R mutation does not restore the catalytic activity of Flag-Dronc\textsuperscript{K78RC318A}. They further suggest that the suppression of the pupal lethality of dronc mutants by expression of Flag-Dronc\textsuperscript{K78RC318A} occurs independently of the catalytic activity of Dronc which is therefore not absolutely essential for the survival of the flies. These data further imply that K78 mono-ubiquitylation controls additional, non-catalytic (apoptosis- and effector-caspase-independent) functions of Dronc whose failure in dronc mutants contribute to lethality.

\textit{K78 ubiquitylation of Dronc is involved in control of apoptosis-induced proliferation}

Next, we examined whether K78 mono-ubiquitylation is involved in a non-apoptotic function of Dronc. We and others have shown that Dronc can trigger apoptosis-induced proliferation (AiP) of neighboring surviving cells independently of downstream effector caspases and thus apoptosis \cite{178, 180, 181, 183}. Expression of the effector caspase inhibitor P35 is used to uncouple AiP from apoptosis. This treatment blocks apoptosis, but triggers chronic Dronc activity which causes tissue overgrowth due to
permanent AiP [177, 178, 180, 183, 306, 307]. It was previously shown that co-expression of p35 with dronc or pro-apoptotic hid using ey-Gal4 (ey>dronc+p35 or ey>hid+p35) in eye imaginal discs causes head overgrowth with pattern duplications, while expression of catalytically inactive ey>dronc$^{C318S}$+p35 did not [177, 178, 183]. Consistently, expression of Flag-Dronc$^{wt}$ and Flag-Dronc$^{K78R}$ in ey>p35 or ey>hid+p35 background induced or enhanced head overgrowth, respectively, while catalytically inactive Flag-Dronc$^{C318A}$ displayed wild-type head phenotypes in these assays (Figure 2.11A, B; Figure 2.12A). Surprisingly, however, expression of Flag-Dronc$^{K78RC318A}$ in ey>hid+p35 and ey>p35 assays also showed a similar overgrowth phenotype compared to Flag-Dronc$^{wt}$ or Flag-Dronc$^{K78R}$ (Figure 2.11A, B; Figure 2.12A). Thus, similar to the results obtained in the rescue crosses of dronc induced lethality, loss of K78 ubiquitylation can suppress loss of catalytic activity in AiP. As controls, we expressed Flag-Dronc constructs with ey-GAL4 in the absence of p35. However, simple overexpression of the Flag-Dronc construct did not trigger any overgrowth phenotype in these crosses (Figure 2.12B).

Because we showed in Figures 2.5 and 2.7 and, that Flag-Dronc$^{K78R}$ interacts better with GFP-Dark than Flag-Dronc$^{wt}$, we wondered if the rescue of AiP by Flag-Dronc$^{K78RC318A}$ is dependent on the interaction with Dark. Indeed, in the absence of Dark (by RNAi), Flag-Dronc$^{K78RC318A}$ is no longer able to restore AiP in ey>hid+p35 background (Figure 2.11A, B).

**K78 ubiquitylation of Dronc is involved in control of male genitalia rotation**
Figure 2.11. K78 ubiquitylation plays inhibitory roles for additional functions of Dronc.

(A) Quantification of the enhanced head overgrowth phenotype of ey>hid+p35 animals expressing the indicated Flag-Dronc transgenes. Overgrowth is characterized by expanded head cuticle with pattern duplications such as bristles and ocelli (see examples in (D)). Flag-Dronc<sup>C318A</sup> acts in a dominant negative manner in ey>hid+p35 background. Flag-Dronc<sup>K78RC318A</sup> phenotype in ey>hid+p35 background is dependent on Dark as observed by 94% suppression of the overgrowth phenotype when dark RNAi is expressed.

(B) Representative head phenotypes of ey>hid+p35 animals expressing the indicated Flag-Dronc transgenes.

(C) Male genitalia rotation defect of dronc null mutants is fully suppressed by da>Flag-Dronc<sup>wt</sup> and da>Flag-Dronc<sup>K78R</sup> (100% of males display 360° rotation) (quantified in B) and partially suppressed by da>Flag-Dronc<sup>K78RC318A</sup> (62% of males display 360° rotation). da>Flag-Dronc<sup>C318A</sup> failed to suppress this phenotype. The i surrounded by a circle indicates the relative orientation of the male genitalia in the depicted animals (i =
wild-type). The suppression by $da>\text{Flag-Dronc}^{\text{K78RC318A}}$ is partially reverted (38% of full rotation) when Dark RNAi is expressed (quantified in B).

**D** Quantification of male genitalia rotation defect phenotype in dronc null mutants, expressing the indicated Flag-Dronc transgenes.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05; ** P<0.01; ns – not significant.
Figure 2.12. Flag-Dronc$^{K78R}$ and Flag-Dronc$^{K78RC318A}$ can induce a head capsule overgrowth phenotype.

(A) Expression of Flag-Dronc$^{wt}$, Flag-Dronc$^{K78R}$ and Flag-Dronc$^{K78RC318A}$ in ey>p35 background can induce overgrowth phenotypes. Overgrowth is characterized by expanded head cuticle with pattern duplications such as bristles and ocelli. In contrast, Flag-Dronc$^{C318A}$ cannot induce this phenotype.

(B) Expression of indicated Flag-Dronc constructs with ey-GAL4 does not lead to any eye phenotype.

For quantifications, the student’s t-test was used. Error bars are SD. * P<0.05; ** P<0.01; ns – not significant.
During development, *Drosophila* male genitalia make a full 360° clockwise rotation [308]. When components of the apoptotic machinery (*hid, dronc, drICE*) are impaired, the rotation fails or is incomplete [309-312] suggesting that it is an apoptosis-driven event. We examined whether expression of da>Flag-Dronc constructs could rescue the genitalia rotation defect in *dronc*124/dronc129 males. da>Flag-Dronc^wt^ and da>Flag-Dronc^K78R^ fully rescued the male genitalia rotation phenotype of *dronc* mutant males (100% of males display 360° rotation) (Figure 2.11C; quantified in Figure 2.11D). In addition, these males were fertile. In contrast, da>Flag-Dronc^C318A^ was unable to rescue the *dronc*124/dronc129 rotation defect and had incomplete rotations ranging from 180° to 270° (Figure 2.11C, D). These males were also sterile. Interestingly, da>Flag-Dronc^K78RC318A^ partially rescued the rotation defect associated with *dronc* null mutations (62% of males display 360° rotation) (Figure 2.11C, D). However, sterility caused by *dronc* null mutations was not suppressed suggesting that other non-apoptotic processes such as sperm maturation are not rescued [206]. The partial rescue of the rotation phenotype by Flag-Dronc^K78RC318A^ is potentially interesting because it may suggest that Dronc has two functions for male genitalia rotation: in addition to the previously reported effector caspase-dependent function [311, 312], it may also have an effector caspase-independent function. Because effector caspases require catalytic activity of Dronc for activation, only the effector caspase-independent function can be rescued by Flag-Dronc^K78RC318A^, giving rise to the observed partial rescue (Figure 2.11C, D). The rescue of the rotation phenotype by Flag-Dronc^K78RC318A^ is also dependent on Dark – at least partially – as *dark* RNAi reduces the rescue to 38% full rotation (Figure 2.11D). These
data further suggest that K78R mutation is an intrinsic suppressor of loss of Dronc’s catalytic activity.

DISCUSSION

Implications of K78 mono-ubiquitylation for apoptotic functions of Dronc

Our in vivo data uncovered an elegant mechanism of how Dronc activation is regulated through mono-ubiquitylation and how this modification affects both catalytic and non-catalytic functions of Dronc. Our MS/LC-MS data from larval and pupal samples demonstrate that in living cells, Dronc is mono-ubiquitylated at K78. Because mono-ubiquitylation is not a mark for proteasome-mediated degradation, this finding explains why monomeric Dronc is not degraded in living cells [89]. Mono-ubiquitylation of Dronc is not an unprecedented observation in the caspase field. It was previously reported that cIAP2 promotes mono-ubiquitylation of the effector caspases Caspase-3 and Caspase-7 in vitro [248]. However, the significance of this mono-ubiquitylation is not known. Furthermore, the paracaspase MALT1 is subject to mono-ubiquitylation [313, 314]. Interestingly, this modification leads to MALT1 activation. Here, we add the initiator caspase Dronc in Drosophila to the list of caspases being mono-ubiquitylated.

Mono-ubiquitylation of K78 of Dronc does not regulate the intrinsic catalytic activity of Dronc. Purified recombinant Flag-Droncw and Flag-DroncK78R have comparable catalytic activities in vitro. However, the location of K78 in the CARD domain suggests a regulatory modification for the interaction with Dark. Consistently, K78 was recently reported to be a critical residue for the interaction between the CARD
domains of Dronc and Dark [95]. Indeed, our genetic analysis suggests that Flag-Dronc<sup>K78R</sup> increases the physical association with Dark, resulting in increased processing of Dronc and thus higher apoptotic activity. Thus, we propose that in living cells, K78 mono-ubiquitylation of Dronc prevents the interaction with Dark.

Because of the increased processing and activation of Flag-Dronc<sup>K78R</sup>, we expected a very strong apoptotic phenotype when expressing Flag-Dronc<sup>K78R</sup> in flies. However, although we observed increased apoptosis by expression of Flag-Dronc<sup>K78R</sup> compared to Flag-Dronc<sup>wt</sup>, it was not as severe as expected and depended on the presence of mis-expressed Dark. There are a few possibilities to explain this result. Although K78 was identified as the only ubiquitin acceptor site by LC-MS/MS analyses, we did not see a complete loss of mono-ubiquitylation in Flag-Dronc<sup>K78R</sup> flies. It is possible that when this major ubiquitin acceptor site is mutated, another Lys residue is selected for ubiquitylation. Nevertheless, the partial loss of ubiquitylation in Dronc<sup>K78R</sup> (Figure 2.2E) is sufficient to shift Dronc activity to a higher level. This increased activity depends on the presence of Dark.

Another possibility to explain the absence of a significant apoptotic phenotype of da>Flag-Dronc<sup>K78R</sup> is that correct stoichiometry between Dronc and Dark molecules is important for execution of apoptosis [99]. These proteins mutually control their stability. Overexpression of one protein triggers degradation of the other one [99]. This balance ensures that the levels of functional apoptosome units are low and this is most likely the reason why expression of each protein by itself in a tissue or even in the whole animal does not cause a significant apoptotic phenotype or complete lethality [99]. Only if both
proteins are co-expressed can a significant apoptotic phenotype be recorded and under those conditions can Flag-Dronc<sup>K78R</sup> trigger a stronger apoptotic phenotype compared to Flag-Dronc<sup>wt</sup>, as observed in Figure 2.5.

**Implications of K78 mono-ubiquitylation for non-catalytic functions of Drone**

We also examined the role of K78 ubiquitylation in a catalytically inactive (C318A) Dronc background. da>Flag-Dronc<sup>C318A</sup> fails to rescue any of the dronc null mutant phenotypes examined such as lethality, apoptosis and male genitalia rotation, and also fails to induce AiP. However, surprisingly, the ubiquitylation-defective and catalytically inactive double mutant of Dronc (da>Flag-Dronc<sup>K78RC318A</sup>) does rescue the lethality and male genitalia rotation phenotypes of dronc null mutants and promotes AiP (Figures 2.8 and 2.10). The rescue of these phenotypes is not the result of restoring the catalytic activity of Flag-Dronc<sup>K78RC318A</sup> by the K78R mutation because in vitro cleavage assays demonstrated that the effector caspase drICE was not processed and in vivo IOC apoptosis was not rescued in dronc null mutants (Figure 2.8A, B; Figure 2.9), indicating that Flag-Dronc<sup>K78RC318A</sup> has no catalytic and thus no apoptotic activity. Therefore, even though Flag-Dronc<sup>K78R</sup> is released from inhibitory ubiquitylation, it still needs its catalytic activity to execute apoptosis. Flag-Dronc<sup>K78RC318A</sup> is an intragenic suppressor of several, but not all, phenotypes associated with loss of the catalytic activity of Dronc. Therefore, the Flag-Dronc<sup>K78RC318A</sup> transgene offers unique opportunities to identify and characterize apoptosis- (effector caspase-) independent functions of Dronc and to distinguish them from effector caspase-dependent ones.
These results allow making the following important conclusions about Dronc function. Firstly, the pupal lethality (which is actually a strong semi-lethality) associated with *dronc* null mutations is not only due to loss of the catalytic (enzymatic) activity. It appears that some non-catalytic functions of Dronc are also very important for survival of the animal. Loss of the catalytic activity may contribute to the pupal lethality, but it may not be the underlying cause. This conclusion may not apply to the embryonic lethality of *dronc* germline clones [61]. Secondly, because we demonstrated that K78 mono-ubiquitylation controls the interaction of Dronc with Dark, it appears that Flag-Dronc*K78RC318A* executes its non-enzymatic functions also through increased interaction with Dark. Thus, increased interaction with Dark is sufficient for induction of several non-apoptotic functions of Dronc such as AiP. Thirdly, it is a hot debate in the caspase field how caspases are restrained from inducing apoptotic death during non-apoptotic processes [315-317]. However, our results imply that at least for the caspase Dronc, its catalytic activity is not strictly required for non-apoptotic processes, although it may contribute to it. Instead, it appears that K78 mono-ubiquitylation controls activation of Dronc for non-apoptotic processes without requiring the catalytic function of Dronc.

**Evolutionary considerations**

Dronc is considered to be the *Drosophila* Caspase-9 ortholog; however, it has more protein similarity to mammalian Caspase-2 [58]. Alignment of the CARD domains of Dronc and Caspase 2 showed that K78 is not a conserved residue. However, there are two conserved Lys residues at positions 20 and 65. It is possible that Caspase-2 may be ubiquitylated at one of these residues and this ubiquitylation may play a role in formation
of the PIDDosome, an apoptosome-like protein complex required for Caspase-2 activation [318]. On the other hand, Caspase-9 does not have any Lys residue in its CARD domain. It is possible that the CARD domain of Caspase-9 has not evolved an ubiquitylation control mechanism because the interaction between Caspase-9 and Apaf-1 is not rate limiting for Caspase-9 activation (Cytochrome c release is). Nevertheless, similar to Dronc, mature Caspase-9 ubiquitylation has been shown in vitro [240], suggesting that Caspase-9 activation may be controlled by ubiquitylation after activation in the Apaf-1 apoptosome.

Our work highlights a mechanism where Dronc’s activity is negatively regulated through mono-ubiquitylation that interferes with its interaction partner Dark. This work may help understanding the similarities and differences of caspase activation in mammalian and Drosophila apoptosomes.

**Experimental Procedure**

**Immunoprecipitations and immunoblotting**

Embryos, 3rd instar wandering larvae, 1-2 days old pupae and heads of adult flies were lysed in 100 ul of SDS lysis buffer containing 2% SDS, 150 nM NaCl, 10 mM TrisHCl, 20 uM NEM and protease inhibitors (Promega), respectively. The samples were sonicated for 10 seconds twice after they were boiled at 100°C for 10 minutes. 900 ul of dilution buffer (10 mM TrisHCl, 150 mM NaCl, 2 mM EDTA and 1% Triton-X) was added to the samples and samples were rotated at 4°C for 1 hour before centrifugation for 30 minutes. Protein concentrations of supernatants were measured by Bradford Assay. 30
ug and 425 ug of total protein were used for western blots and IPs, respectively. IP was performed with anti-Flag M2 magnetic beads (Sigma-Aldrich M8823) overnight at 4°C with rocking. 100 ul of 150 ng/ul Flag peptide in TBS was used for elution which took place at 4°C for 2 hours. 25 ul of eluted protein was used for western blotting. Dilutions of antibodies used are as follows: anti-Flag M2 antibody (1:1000), FK2 and FK1 (Enzo Life Sciences – 1:200), anti-Actin (Millipore Mab1501- 1:2000).

For ubiquitin pull-down assays, 3rd instar larvae were collected and lysed in urea lysis buffer containing 8 M Urea, 0.1 M NaH2PO4, 0.01 M TrisHCl, 0.05% Tween 20, pH 8.0 and protease inhibitors. IP was performed with Nickel-NTA magnetic agarose beads (Qiagen 36111) at 4°C overnight with rocking. 60 ul of 250 mM of Imidazole in urea lysis buffer (pH 4.5) was used for elution. 30 ul of eluted protein was analyzed by western blot. Anti-His antibody (Thermo Scientific-Fisher MA1-21315) was used at 1:1000 dilution.

For co-IPs, 3rd instar larvae were collected and lysed in NP40 buffer (20 mM TrisHCl pH 8.0, 137 mM NaCl, 1% NP40, 2 mM EDTA and protease inhibitors). IP was performed with GFP-Trap (ChromoTek) magnetic beads at 4°C overnight. GFP-Dark protein was eluted with 50 ul of 0.2M Glycine buffer pH 2.5. 25 ul of eluted protein was used for western blot. Anti-GFP antibody (Thermo Scientific-Fisher MA5-15256) was used at 1:200 for IP-western blots, 1:1000 for western blots.

Immunoblot band intensities are quantified with GelQuantNET software provided by biochemlabsolution.com.
Deubiquitylation Assay

Immunoprecipitated Flag-Dronc was incubated with 3 ul of USP2 enzyme (Boston Biochem E-504) in deubiquitylation assay solution (50 mm EDTA, 100 mm DTT, 50 mm Tris-HCl and 150 mm NaCl) for 90 min at 37°C.

LC-MS/MS Analysis

Flag-Dronc was immunoprecipitated from larval and pupal da>Flag-Dronc extracts as described above. 1 mg of protein was used for IPs. Elutions of eight IPs were pooled and concentrated with 0.5 ml centrifugal tubes (Millipore UFC500324). In vitro ubiquitylation assays were performed as described previously [88]. Concentrated IP samples and in vitro ubiquitylated Dronc were loaded to 4-20% gradient SDS-PAGE gels. The gels were stained with Coomassie Blue Solution (Thermo Scientific-Fisher-24590) and the 60 kDa band as well as higher molecular weight bands (for in vivo samples) were excised and submitted to MS Bioworks (Ann Arbor, MI). Samples were digested with Chymotrypsin and analyzed by LC-MS/MS. In the in vitro and in vivo samples, one peptide (K78ITQRGPTAY) carried the di-Glycine motif, characteristic for ubiquitylation.

Fly Work and Generation of Transgenic Flies

The following fly stocks were used: daughterless (da)-Gal4; GMR-Gal4; UAS-Flag-Dronc [98]; UAS-Flag-Droncw; UAS-Flag-DroncK78R, UAS-Flag-DroncC318A and UAS-Flag-DroncK78RC318A (this work); UAS-6xHis-ubiquitin (this work); UAS-GFP-Dark, UAS-GFP-DarkV and UAS-GFP-DarkCC [99]. droncP24 and droncP29 [61]; ey>p35 and
Please note that two $UAS$-$Flag$-$Drone^{wt}$ transgenes were used. The first one (a kind gift of Dr. Sally Kornbluth) was used in the initial phases of this work and has a random insertion on chromosome 3 [98]. The second one was obtained by phiC31 site-specific integration in the VK37 landing site on chromosome 2 (see below). This line was used in combination with $UAS$-$Flag$-$Drone^{K78R}$, $UAS$-$Flag$-$Drone^{C318A}$ and $UAS$-$Flag$-$Drone^{K78RC318A}$. All crosses were carried out at room temperature. 3L MARCM clones were induced by heat shocking L1 larvae at 37°C for 45 minutes as described [319]. Co-expression of $UAS$-$GFP$-$Dark$ and $UAS$-$Flag$-$Drone$ transgenes was controlled by GAL80$^{ts}$ [320]. Temperature shift was performed at 29°C for 24 h. 3rd instar larvae were collected for lysis immediately after temperature shift.

Wild-type and mutant $UAS$-$Flag$-$Drone$ transgenic flies were generated by the phiC31 site-specific integration system [304, 305]. Flag-Dronc-pTFW and Flag-Dronc$^{C318A}$-pAFW vectors were kind gifts from Dr. Sally Kornbluth. Flag-Dronc and Flag-Dronc$^{C318A}$ were cloned into pENTR3C vector. Point mutations were generated by site-directed mutagenesis. AttB site for site-specific integration was cloned into pTFW vector (DGRC - 1115). Wild-type and mutant Flag-Dronc coding sequences were cloned into attB-pTFW vector by Gateway Cloning Technology (Gateway LR Clonase II Enzyme Mix). Plasmids were sent to Genetivision for injection. VK37 landing site was used for phiC31 integration [321].

$UAS$-$6xHis$-$Ubiquitin$ transgenic flies were generated by random integration (Bestgene) of a pUAST-$6xHis$-$Ubiquitin$ construct created by inserting a KpnI-XbaI fragment of N-terminal 6xHis human Ubiquitin pcDNA3.1 into pUAST [302].
Expression of 6xHis-Ubiquitin was validated by FK2 Western blotting of urea-based lysis/Ni\(^{2+}\)-based purification lysates generated from 20 adult da-GAL4;UAS-6xHis-Ubiquitin flies.

**Immunohistochemistry**

3\(^{rd}\) instar larval brain lobes with eye discs were dissected in PBS and fixed in 4% PFA. Samples were blocked with 2% NDS in PBST and stained with c-Dcp-1 (Cell Signaling 9578- 1:100) and anti-Flag (1:200) antibodies [322]. TUNEL was performed as described [323]. For pupal dissections, pupae were aged to 42 h-48 h APF. Pupal discs were dissected, fixed and stained for c-Dcp-1 and Dlg (DSHB 4F3 anti-disc large -1:100) [322]. Imaginal discs were mounted in Vectashield and imaged by confocal microscopy.

**Caspase Activity Assays**

Caspase activity assays were performed as described [323, 324]. Briefly, adult heads were lysed in caspase assay buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM DTT, 0.5% TritonX-100, 4% glycerol and protease inhibitors). Protein concentration was measured with Bradford Assay. 40 ug of protein was incubated with 100 uM of DEVD-AMC caspase substrate (MP Biomedicals 195868) in a final volume of 100 ul of caspase assay buffer. Fluorescence was measured with spectrophotometer (excitation 385 nM emission 460 nM) at 15 min intervals for 3 hours at 37°C. Each experiment was done at least three times.

**Caspase Cleavage Assay**
For *in vitro* cleavage assays, wild type and mutant Dronc coding sequences were cloned into pET-28a plasmid to yield 6xHis fusion proteins. Generated plasmids were transformed to BL21(DE3)pLysS competent cells (Promega L1191). 50 ul of bacterial culture was grown at 37°C. Plasmid expression was induced by 0.2 mM IPTG for 3 h at 30°C as described [325]. Bacterial pellets were lysed with 4 ml of CellLytic B Cell Lysis Reagent (Sigma-Aldrich B7435) after adding 0.2 mg/ml Lysozyme, 50 units/ml Benzonase and 1X protease inhibitor (Roche).

drICE<sup>C211A</sup>-pET23b plasmid was a kind gift from Dr. Guy Salvesen [97]. drICE<sup>C211A</sup> coding sequence was cloned into PT7CFE1-Nmyc plasmid (Thermo Scientific 88863). Myc-drICE<sup>C211A</sup> protein was generated by using TNT Rabbit Reticulocyte Lysate System (Promega L4610). 4 ul of Myc-drICE<sup>C211A</sup> protein was incubated with 100 ug of wild-type and mutant 6xHis-Dronc protein in caspase assay buffer (100 mM Hepes pH 7.5, 0.1 % CHAPs, 10% sucrose, 10 mM DTT, 50 mM Nacl, 0.5 mM EDTA, protease inhibitor). The reaction was incubated at 30°C for 3 hours [96] and analyzed by western blotting. Anti-Myc antibody (Santa Cruz SC40) was used at 1:200 concentration.

**Statistical Analyses**

Student’s t-test is used in all graphical analyses with parametric statistics. Crosses are repeated at least three times. Numbers of fly eyes used for area calculation and staining intensity are indicated in corresponding figures. The quantification of eye size was done using the Histogram function in Photoshop.
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CHAPTER III

Conclusions and Future Directions

Part I. Conclusions

The ubiquitin system generally correlated with cell death. In particular, ubiquitylation has been shown to control stability and activity of pro- and anti-apoptotic proteins. This control is crucial as even a slight deflection could have catastrophic results for cells. This thesis study provides a great example of how activity of an initiator caspase Dronc could be regulated by ubiquitylation without causing degradation and how this ubiquitylation is important for both apoptotic and non-apoptotic functions of this initiator caspase Dronc.

As opposed to what previous in vitro studies suggested [78, 80, 81], our study demonstrated that Dronc is mono-ubiquitylated throughout development from embryonic stages to adulthood. Persistence of this ubiquitylation throughout development could hint its importance for Dronc’s regulation. Incubating IPed Flag-Dronc with USP2 deubiquitinase removed this ubiquitylation almost completely. Interestingly, although we expected to observe a size difference between ubiquitylated and un-ubiquitylated Flag-Dronc, we could not observe this after removing mono-ubiquitin on Dronc with USP2. Because our results suggested that Dronc is mono-ubiquitylated, which is a non-degradative mark, we thought of a few ways how ubiquitylation controls Dronc.
Ubiquitylation of Dronc could affect the interaction with Dark, its dimerization or its catalytic activity. Further examination of this ubiquitylation through mass-spectrometry analysis identified K78 in the CARD of Dronc as the site of ubiquitylation and suggested a role for its interaction with Dark. Generating a K78R mutant Dronc (Flag-Dronc\textsuperscript{K78R}) resulted a significant reduction of mono-ubiquitylation. Although K78 is the only residue shown to be ubiquitylated by mass-spec analyses of Flag-Dronc\textsuperscript{wt}, we could still see residual ubiquitylation of Flag-Dronc\textsuperscript{K78R}. Dronc has 32 lysine residues. It is possible that the ubiquitin machinery could act on another lysine when K78 is mutated. Nonetheless, K78 is the major ubiquitin acceptor site.

Understanding the function of this ubiquitylation is crucial for Dronc’s activity because mono-ubiquitylation is not degradative and there are not many examples for caspases regulated by mono-ubiquitylation. In fact, the ubiquitylation status of most mammalian caspases is still unclear. Thus, understanding this regulatory ubiquitylation could help us to extend our knowledge to other caspases that could have similar regulations. A previous study suggested that separate expression of each apoptosome component in the Drosophila eye does not lead to any phenotype; however, simultaneous expression of both leads to small and deformed eyes [99]. In the light of this information, we analyzed expression of mutant Dronc and Dark in the Drosophila eye. Expression of Flag-Dronc\textsuperscript{K78R} with GFP-Dark showed smaller and more deformed eyes than expression of Flag-Dronc\textsuperscript{wt} and GFP-Dark due to increased apoptosis. A caspase activity assay further confirmed the observed results that Flag-Dronc\textsuperscript{K78R} has more activity when co-expressed with GFP-Dark. In addition, \textit{in vitro} cleavage assay with Myc-drICE\textsuperscript{C211A}
showed that the enhanced activity of Flag-Dronc\textsuperscript{K78R} does not result from alterations in its catalytic activity. Co-IP experiments supported the genetic data that Flag-Dronc\textsuperscript{K78R} could interact with GFP-Dark better than Flag-Dronc\textsuperscript{wt} because inhibitory ubiquitylation is removed in Flag-Dronc\textsuperscript{K78R}. This enhanced interaction resulted in a better autocleavage of Flag-Dronc\textsuperscript{K78R} than Flag-Dronc\textsuperscript{wt} and was the reason for increased apoptotic labeling in the larval eye discs. In support of these findings, a recent structural study showed that K78 forms an intramolecular H-bond with a critical residue (Q81) to enhance the interactions between the CARDs of Dronc and Dark in the apoptosome. All in all, we conclude that K78 mono-ubiquitylation of Dronc is indeed inhibitory of Dronc’s activation due to its impaired interaction with Dark (Figure 3.1).

Previous studies conducted \textit{in vitro} showed that DIAP1 ubiquitylates Dronc \cite{78, 80, 89}. We tested this possibility \textit{in vivo} and observed that heterozygosity at \textit{diap1} decreased mono-ubiquitylation of Dronc, suggesting that DIAP1 mono-ubiquitylates Dronc \textit{in vivo}. Next, we sought to identify if DIAP1 ubiquitylates Dronc at K78. For this we used two different approaches. In our first approach, we performed \textit{in vitro} ubiquitylation assays with Dronc, DIAP1 and Ubcd1 or its human homolog UBE2D2 and submitted it for mass-spectrometry analysis. These analyses indicated that DIAP1 can ubiquitylate Dronc at K78. Secondly, loss of one functional copy of \textit{diap1} enhanced the eye phenotype of GMR-driven GFP-Dark and Flag-Dronc\textsuperscript{wt} much more than expression of GFP-Dark and Flag-Dronc\textsuperscript{K78R}. Our results conclude that DIAP1 mono-ubiquitylates Dronc at K78 \textit{in vivo} (Figure 3.1).
When K78 inhibitory ubiquitylation is released by K78R mutation, we expected cell lethality of Flag-Dronc\textsuperscript{K78R} flies due to uncontrollable caspase activity and generated a cautionary construct, which was both non-ubiquitable and catalytically inactive (Flag-Dronc\textsuperscript{K78RC318A}). By examining Flag-Dronc\textsuperscript{K78RC318A}, we made a surprising discovery.

Very interestingly, ubiquitous expression of Flag-Dronc\textsuperscript{K78RC318A} could rescue the lethality caused by dronc null mutations, whereas Flag-Dronc\textsuperscript{C318A} alone could not rescue lethality. Investigation of Flag-Dronc\textsuperscript{K78RC318A} in a developmental apoptosis setting such as interommatidial cell (IOC) death in pupal eye discs showed that Flag-Dronc\textsuperscript{K78RC318A} could not rescue extra IOC phenotype in dronc null mutants, suggesting that loss of K78 ubiquitylation does not restore the catalytic activity of Flag-Dronc\textsuperscript{K78RC318A}. These findings support two important conclusions: first, the catalytic activity of Dronc may not be essential for survival of the fly and second, removal of the inhibitory ubiquitylation of Dronc could bypass the requirement for its catalytic activity. Therefore, Dronc’s non-apoptotic functions appear to be more important for the survival of the fly than its apoptotic function.

We tested Flag-Dronc\textsuperscript{K78RC318A} in two different experimental settings where Dronc is known to have apoptosis-independent functions. In the first approach, we examined Flag-Dronc\textsuperscript{K78RC318A} in AiP. Interestingly, expression of Flag-Dronc\textsuperscript{K78RC318A} in ey>hid+p35 background resulted in significantly enhanced overgrowth, which could be suppressed by downregulation of Dark. Secondly, we analyzed male genitalia rotation defects observed in dronc null mutants. Surprisingly, Flag-Dronc\textsuperscript{K78RC318A} could rescue
Figure 3.1. Regulation of Dronc’s activity through mono-ubiquitylation.

DIAP1 mono-ubiquitylates Dronc at K78 which resides in Dronc’s CARD domain. This ubiquitylation interferes Dronc’s interaction with Dark and thus apoptosome formation. As apoptosome formation is required for Dronc activation, K78 ubiquitylation has inhibitory implications for both apoptotic and non-apoptotic functions of Dronc. It is possible that K78 ubiquitylation may block interactions with other proteins that may have an effect in Dronc’s non-apoptotic functions.
male genitalia rotation defects by 62%, which could be reverted to 38% by expression of dark RNAi. We conclude that K78 ubiquitylation also plays an inhibitory role in AiP and male genitalia rotation, suggesting the importance of K78 ubiquitylation in Dronc’s non-apoptotic functions. These findings also revealed that the non-apoptotic functions of Dronc$^{K78\text{RC318A}}$ could be Dark-dependent (Figure 3.1).

**Part II. Discussion and Future Directions**

This thesis study uncovered a regulatory link between the ubiquitin system and apoptosis. Caspases require a tight control due to the nature of their protease activity. This control may involve proteasomal degradation or non-degradative means of inhibition through the ubiquitin system. Our studies showed that Dronc could be mono-ubiquitylated, which is a non-degradative mark. Site of the mono-ubiquitylation resides in Dronc’s CARD domain at K78. K78 mono-ubiquitylation functions to block Dronc’s interaction with Dark, which is essential for formation of the apoptosome and activation of Dronc. Further examination of mono-ubiquitylation of Dronc unraveled its importance for two apoptosis-independent functions of Dronc, AiP and male genitalia rotation. However, it is unclear whether K78 ubiquitylation also plays a role in other non-apoptotic functions of Dronc. In addition, the underlying mechanisms for these apoptosis-independent functions are unknown. The following section will discuss aspects of K78 ubiquitylation in both apoptotic and non-apoptotic signaling and its possible relations to its mammalian counterparts.
A. Connecting Links Between K78 Ubiquitylation and Apoptosis

There is a big debate in the Drosophila apoptosis field about how caspase activity could be controlled when activated by the apoptotic stimuli to avoid unnecessary cell death. Some studies suggest that the apoptosome in complex with DIAP1 is formed in tolerable amounts and when apoptotic stimuli arrive, its activity is amplified [99]. However, when the job is done, “feedback inhibition” and DIAP1 keep caspase activity in control to go back to normal. Specifically, “feedback inhibition” system suggested that Dark and most likely an active, proteolytically cleaved Dronc undergoes degradation potentially through DIAP1. On the other hand, there is evidence of increased transcriptional activity of caspases as a response to apoptosis induction [16]. Furthermore, in the case of effector caspases, unwanted caspase activity could be inhibited after they are activated [88].

K78 ubiquitylation of Dronc is important for answering the question of how initiator caspases are kept inactive; however, it could be contradictory to the “feedback inhibition” theory. It is possible that both scenarios could work. For example, K78 ubiquitylation could be an extra control step. At first step, Dronc activity is inhibited through K78 ubiquitylation but if surpassed, in the second step, apoptosome could be regulated through “feedback inhibition”. Then the next question would be how the first step could be surpassed? This could be regulated through the ubiquitin system such as a specific deubiquitinase that could determine release of K78 ubiquitylation on Dronc so that it could be recruited to the apoptosome. It is possible that after the first step is
surpassed, caspase activity is achieved and could be turned off once again through “feedback inhibition” to avoid further cell death at the end.

Et deubiquitinase was discovered to carry out both anti- and pro-apoptotic functions through long and short isoforms [279]. Induction of apoptosis by the short isoform could be suppressed by upregulation of DIAP1 or downregulation of Dronc but not by P35 expression. This suggests that the function of Et may be exerted through Dronc, but not the effector caspases. Et was shown have homology to mammalian USP36, USP42 and USP17. As an example for the function of a mammalian homolog of Et, USP36 could remove c-Myc and SOD2 poly-ubiquitylation for their stabilization [326, 327]. Furthermore, two recent studies suggested that USP42 could remove poly-ubiquitylation of P53 for its stabilization and mono-ubiquitylation of Histone H2B for activation of gene expression [328, 329]. In addition, USP17 was indicated to remove both K48- and K63-linked poly-ubiquitin chains from substrates [330, 331]. Although there is no evidence of functional specificity of Et, its mammalian homologs suggest that Et may hydrolyze both poly- and mono-ubiquitin. Thus, it is possible that K78 ubiquitylation of Dronc could be removed by Et to mediate apoptosis induction and/or to surpass the first step of the control mechanism.

As DIAP1 being a short-lived and its target Dronc being a long-lived protein, it is intriguing how DIAP1 keeps Dronc ubiquitylated at K78. Because mono-ubiquitylated Dronc is the major form we observed in vivo. DIAP1 was shown to undergo N-end rule degradation. When the first 20 aa of DIAP1 are removed, it is a more stable protein and can bind to UBR proteins to enhance ubiquitylation of effector caspases. It is possible
that binding to caspases blocks DIAP1’s interaction with RHG proteins. Thus, DIAP1 could be a more stable and efficient E3 ligase toward effector caspases. It will be interesting to investigate whether K78 ubiquitylation also involves UBR proteins. Apoptosis induced by downregulation of UBR3 in the *Drosophila* eye could be suppressed by homozygosity at *dronc*, which may suggest that UBR3 may be involved in Dronc’s ubiquitylation at K78. There are 5 other UBR-box containing E3 ligases in the *Drosophila* genome. Testing each of them for K78 ubiquitylation through *in vitro* ubiquitylation and genetic interaction assays will be uncovering their involvement.

Possible degradation of an active, processed Dronc in the apoptosome by DIAP1 is what the “feedback inhibition” theory suggested [99]. It is possible that, as a second control mechanism, in case the first control mechanism is surpassed, Dronc and Dark could be poly-ubiquitylated for its degradation by DIAP1 while in the apoptosome. In fact, expression of both apoptosome components resulted in reduced Dark levels and Dark poly-ubiquitylation (preliminary observation). This is an interesting observation because it supports the “feedback inhibition” theory. It is compelling to find out the site of ubiquitylation and whether DIAP1 is responsible of this event. Because cleavage of Dark at residue 1292 may expose Lysine residues to be poly-ubiquitylated for degradation. Although, Dronc levels are also reduced, there was not any detectable Dronc poly-ubiquitylation in this context (preliminary observation).

In addition to the idea of Dark ubiquitylation in the apoptosome, it will be interesting to investigate any possible ubiquitylation of free Dark for a tighter control of the apoptosome formation. Interestingly, alignment of the CARD domains of Dark and
Dronc shows that K78 is conserved in Dark (K93). This is a residue close to Y85 which forms a critical H bond with a critical residue (R82) in Dronc for apoptosome formation [95]. In addition, atomic resolution of the Dark-Dronc complex unraveled K86 of Dark’s CARD interacting with N92 of Dronc’s CARD through Hydrogen bond, which makes K86 another candidate for regulation of the apoptosome through ubiquitylation [95]. It is intriguing to study whether Dark is ubiquitylated at these residues or others to potentially regulate apoptosome formation.

As seen in male genitalia rotation context, Flag-Dronc<sup>K78RC318A</sup> could rescue rotation defect by 62% and this phenotype could be reverted by only 38% when Dark is downregulated. This suggests that Flag-Dronc<sup>K78RC318A</sup> could exert this function partially independent from Dark. In the case of AiP, Dark downregulation suppressed 100% of Flag-Dronc<sup>K78RC318A</sup>-induced overgrowth. It is still a mystery how a catalytically inactive protein could induce such an effect. It is possible that removing the inhibitory mono-ubiquitylation makes Dronc more susceptible for protein-protein interactions. This could result in recruitment of other initiator caspases or mediating its interaction with scaffolding proteins to recruit Dronc to caspase-rich areas in the cell. To answer these questions, it will be best to do an interactor mass-spectrometry analysis to find out interaction partners of Flag-Dronc<sup>wt</sup>, Flag-Dronc<sup>K78R</sup>, Flag-Dronc<sup>K78RC318A</sup> and Flag-Dronc<sup>C318A</sup>. This analysis may uncover scaffolding proteins such as Crinkled and/or caspases containing CARD domains. It could be also interesting to see novel or annotated UBD proteins that associate Dronc. Interestingly, a structural study of the Drosophila apoptosome showed that Dronc CARD (55-67) could interact with Dark WD1 (597-965)
[95]. This suggests that the CARD domain could interact with WD40 repeats. Thus, it is also possible to see WD40 repeat containing proteins in an interactor assay. Identified interactors could later be tested for their potential involvement in apoptosis or non-apoptotic functions of Dronc.

Post-translational modifications (PTM) are important for fine-tuning of protein activity. There are not many studies on regulation of Dronc through PTMs. In fact, there has been only one study which was about regulation of Dronc through inhibitory phosphorylation as a response to increased NADPH levels [98]. Caspase-9 and caspase-2 were also shown to be phosphorylated at multiple sites. Our mass-spectrometry analyses on Dronc to find out ubiquitylated sites uncovered additional phosphorylation sites other than the reported one. These phosphorylation sites reside at the beginning of the caspase domain of Dronc. Dronc can be cleaved to release its caspase domain from its CARD domain [95-97], which enhances its activity. These phosphorylation sites could potentially regulate cleavage of Dronc to release the caspase domain and to fine-tune its activity. Surprisingly, the same mass-spec analyses also indicated multiple methylation sites for Dronc. Although protein methylation was observed in the cell death field such as inhibitory mono-methylation of P53, there has not been any caspase reported to be methylated [332]. In a mammalian study, Numb was shown to be methylated at its PhosphoTyrosine Binding (PTB) domain, which blocks its interaction with P53 and thus apoptosis of breast cancer cells [333]. A recent study showed that Drosophila epithelial tissue regeneration is impaired when methionine metabolism is altered [334]. In addition, metabolic profiling of hemolymph of dark hypomorph showed reduced methylation
index due to a reduction of S-Adenosyl Methionine (SAM) level [335]. On the other hand, increased SAM level was associated with aging of flies [336]. There is clearly regulation of cellular pathways by methionine metabolism in *Drosophila* that are yet to be discovered. It will be very interesting to see if Dronc is involved in this process. Studying the methylation sites of Dronc observed in the mass-spec analysis and identifying their function will shed light on potential apoptotic or non-apoptotic functions of Dronc in methionine metabolism.

In *Drosophila* testis, during spermatogenesis, spermatogonial cysts undergo cell death before entering meiosis, which is called Germ Cell Death (GCD) [337]. Interestingly, *dronc* null mutant showed a significant decrease in GCD, whereas *dark* mutant did not display any decrease in GCD, suggesting Dronc’s function is required independently of Dark in this process [337]. In addition, effector caspases were not essential for GCD [337]. It is intriguing to test whether Flag-Drone$^{K78R}$ and Flag-Drone$^{K78RC318A}$ could function in GCD. Dronc’s activation mechanism has not been investigated in this context. Examining the role of K78 ubiquitylation of Dronc in this process could clarify if Dronc’s activity and/or presence is required for GCD.

**B. Potential Roles for K78 Ubiquitylation in Non-Apoptotic Systems**

The rescue of lethality of *dronc$^{I24}$/dronc$^{I29}$* flies with expression of catalytically inactive and non-ubiquitable Flag-Drone$^{K78RC318A}$ was unexpected and fascinating. This observation strongly suggests that K78 ubiquitylation may exert its function independently of Dronc’s catalytic activity which seems to be dispensable for survival.
Further examination of this phenomena in AiP uncovered an inhibitory role for Flag-Dronc\textsuperscript{K78RC318A}. In addition to AiP, Dronc has other distinct non-apoptotic functions in different systems (discussed in detail in Part ID2). Flag-Dronc\textsuperscript{K78RC318A} construct carries a great importance for discovering mechanisms for its non-apoptotic functions that do not require its catalytic activity such as neuroblast differentiation and border cell migration. Flag-Dronc\textsuperscript{K78R} and Flag-Dronc\textsuperscript{K78RC318A} constructs could also help identifying mechanisms underlying non-apoptotic functions of Dronc that require its catalytic activity such as SOP development, neural pruning and spermatid individualization.

A recent study identified Numb as an interactor of Drone in Type II neuroblasts in the \textit{Drosophila} Central Nervous System (CNS) \cite{200}. Specifically, the PTB domain of Numb which includes conserved phosphorylation sites and the small subunit of Dronc are essential for this interaction \cite{200}. Furthermore, phosphorylation of Numb impairs its interaction with Dronc. Numb localizes and inhibits Notch signaling in differentiating neurons \cite{200}. Not localization but its function was impaired by phosphorylation of Numb, eliminating its tumor suppressor role \cite{200}. Interestingly, expression of catalytically inactive Dronc could suppress the overproliferative neuroblast phenotype similar to wild-type Dronc by initiating neuronal differentiation \cite{200}. This suggests that interaction of Numb and Dronc may not result in cleavage of Numb but may involve a signaling event downstream which is required for neural differentiation. Numb could inhibit Notch signaling through recruiting endocytic machinery for receptor-mediated endocytosis of Notch \cite{338}. In another mechanism, Numb could bind to Sanpodo (a transmembrane protein that interacts with NICD for its activation) to prevent its...
localization to the plasma membrane [339]. Although CARD of Dronc is not required for its interaction with Numb, it may still serve a function through binding adaptor proteins such as Crinkled for recruitment of Numb to the plasma membrane for its function. It will be interesting to investigate whether K78 ubiquitylation has a role in Numb/Dronc-mediated function. Specifically, testing Flag-Dronc\textsuperscript{K78R} or Flag-Dronc\textsuperscript{K78RC318A} to see whether removal of K78 ubiquitylation could enhance inhibition of Notch signaling together with Numb will be interesting. Similarly, testing involvement of Dark in this process will be intriguing.

In addition to CNS, Numb has also been implicated to function in asymmetric cell division and differentiation of neurons by inhibiting Notch signaling in SOP development [340]. It is unclear whether Dronc could interact with Numb in this context as well. Interestingly enough, Numb carries a Dronc cleavage site (TQTD) outside of its PTB domain. It is possible that Dronc may exert its function in SOP development or other contexts through cleaving Numb.

During \textit{Drosophila} oogenesis, a group of epithelial cells migrate from the anterior end of the egg chamber to the anterior end of oocyte (egg chamber stage early 9-10), which is called border cell migration [201]. There are many regulators of this process. For example, Rac GTPase could control actin rearrangement during migration [201]. A dominant negative form of Rac (RacN17) was shown to inhibit border cell migration, which could be rescued by overexpression of DIAP1 [203]. This phenotype could be further rescued by dominant negative form of Dronc or hypomorphic \textit{dark} mutants, but
not by P35 expression [203]. This suggest that border cell migration is negatively regulated by the apoptosome, but not effector caspases. In this context, Dronc may cleave proteins that are downstream of Rac activity such as actin cytoskeleton components to impair border cell migration. Testing Dronc’s involvement in this context by examining its expression levels with Dark in the egg chambers, its possible interactions with and its activity toward other players of border cell migration will be intriguing. In addition, investigating whether Flag-Dronc\textsuperscript{K78RC318A} could rescue Rac17-induced phenotype better or worse than Flag-Dronc\textsuperscript{C318A} will be interesting. Moreover, testing defects after expression of UAS-Dronc constructs including Flag-Dronc\textsuperscript{K78RC318A} in the migrating cells could indicate surprising results. Slow border cells (Slbo) is a transcription factor that is required to turn on activation of motility effectors such as E-cadherin in border cells between Stages 8-9 [201]. Expression of Slbo is controlled by the JAK/STAT pathway. Overexpression of Slbo did not rescue RacN17-induced impairment of cell migration, suggesting Slbo could be upstream of or in parallel to Rac activity [201]. Interestingly, Slbo has a Dronc cleavage site (TQTD). Potential cleavage of Slbo by Dronc may interfere with its activation in a parallel pathway to Rac-DIAP1-Dronc-Dark pathway.

\textit{Drosophila} bristles originate from a single cell called Sensory Organ Precursor cell through asymmetric divisions and differentiation [341]. This process occurs between late larval to early pupal stages and involves various players and signaling events [341]. One of these players is Shaggy46 which regulates Wg signaling negatively [189]. Interestingly, Shaggy46 was shown to interact with Dronc, which facilitates its cleavage by effector caspases [189]. Further investigation showed that Crinkled acts as a
scaffolding protein to recruit Shaggy46 to caspases and mediate its cleavage by effector caspases [188]. Shaggy46 cleavage is essential for its kinase activation and inhibition of Wg signaling [189]. Because Dark and activation of Dronc are required for this process, it will be interesting whether the Flag-Dronc\textsuperscript{K78R} mutant could provide a better cleavage hence increased activation of Shaggy46 due to its enhanced interaction with Dark. This may even lead to missing bristles on the notum. Another consideration would be testing Flag-Dronc\textsuperscript{K78RC318A} in this context to see whether Shaggy46 could still be activated as K78R mutation was found to be intrinsic suppressor of catalytic inactivity in other contexts.

Another context where both initiator and effector caspases are required for tissue remodeling is dendrite pruning. During metamorphosis, dendrites of C4da neurons are eliminated without damaging the neuron itself between 7-12 h after puparium formation [192, 193]. Localized caspase activity in dendrites provides this specialized event. Because dronc mutants provided a better protection against dendrite pruning than P35 expression, effector caspases are possibly not the only targets of Dronc in this context [195]. Previous studies mostly focused on upstream regulation of Dronc such as DIAP1. It is compelling to find out the downstream effectors of Dronc for dendrite pruning. Dendrite pruning resembles apoptosis. For example, severing of dendrites occurs before caspase activation and debris is cleared by phagocytosis [342]. There may be common substrates of Dronc in dendrite pruning and canonical apoptosis. Because expression of a dominant negative form of Dronc impairs dendrite pruning, it will be intriguing to examine Flag-Dronc\textsuperscript{K78RC318A} in C4da neurons to see whether the process will be
impaired or not. Moreover, it could be investigated whether Flag-Dronc$^{K78R}$ is more active than Flag-Dronc$^{wt}$ in rescuing defects in neural pruning. In addition, Dark’s role has never been examined in dendrite pruning. It will be interesting to see whether dark mutants also exhibit impairment in this process.

Spermatid individualization is a process occurring in late spermatogenesis. Both initiator and effector caspase activity could be observed in elongated cysts, cystic bulges and waste bags [103, 205]. Individualization Complexes (IC) form close to nuclear ends of elongated cysts and consist of cytoskeletal membrane complexes. IC of each spermatid goes along in great synchronization along the cyst. Expression of a dominant negative form of dronc or dronc null mutants interferes with IC and thus spermatid individualization [103, 205]. My preliminary observation suggests that Flag-Dronc$^{K78RC318A}$ could surprisingly suppress spermatid individualization defects in dronc null mutants. It is a mystery how Flag-Dronc$^{K78RC318A}$ could have such an effect. As suggested for other functions, Flag-Dronc$^{K78RC318A}$ could be interacting with other proteins/caspases to exert its function. It is important to note that sterility due to catalytic inactivity could not be suppressed by removing inhibitory ubiquitylation. The cyt c-d isoform was previously shown to be required for spermatid individualization and caspase activation [103, 105]. It will be interesting to test whether cyt c-d associates with the Drosophila apoptosome during spermatid individualization. dBruce, an E2 conjugase with BIR domain, is also involved in this process. dBruce levels are higher where caspase activity is lower [207]. dBruce null mutants showed condensed and rounded nuclei in the cysts, suggesting excessive caspase activity [103]. dBruce is known to target Reaper and
Grim; however, its function in this context is not clarified. dBruce could potentially target RHG proteins for degradation to inhibit caspases or target caspases directly during spermatid individualization. Examining these scenarios in testes could be interesting, especially in terms of ubiquitylation of Dronc. As a side note, the mammalian dBruce homolog, Apollon was shown to ubiquitylate caspase-9 in mammalian cells [254]. On the other hand, a deubiquitinase, DUBA is required for proper spermatid individualization [280]. It is unknown whether K78 ubiquitylation also occurs in spermatid individualization for controlling caspase activity; however, DUBA may be deubiquitylating Dronc at K78 to remove inhibition during spermatid individualization.

Examining the phenotypes of Flag-Dronc\(^{K78RC318A}\) in above-mentioned systems could take advantage of a more practical system. An effector caspase biosensor such as CaspaseTracker could be useful and efficient for identifying both ongoing and past caspase activity in specific tissue types [343]. For Dronc-only-dependent functions, a Dronc-specific biosensor could be developed. CaspaseTracker sensor relies on endogenous caspase activity [343]. Using UAS-Flag-Dronc constructs may not answer our questions correctly. It will be a good idea to mutate endogenous Dronc at K78 and/or C318A and generate endogenous Flag-Dronc\(^{K78R}\) and Flag-Dronc\(^{K78RC318A}\) fly lines through Crispr/Cas9 technology [344]. These fly lines will more accurately show caspase activity and localization associated with K78R and K78RC318A mutations in these non-apoptotic systems.

C. Connecting Links Between K78 Ubiquitylation and Diseases
Downregulation of apoptosis is commonly seen in cancer cells. Circumventing inhibitory ubiquitylation of caspases could help activating apoptosis machinery in tumor cells. K78 residue is unfortunately not conserved in caspase-9. In fact, caspase-9 does not have any Lysine residues at its CARD domain. However, caspase-2 which has 46% similarity to Dronc have multiple Lysine residues at its CARD domain. Investigation of caspase-2 ubiquitylation status at its CARD domain could reveal a controlling mechanism for its activation, similar to Dronc. Lack of caspase-2 was shown to accelerate oncogene-driven tumorigenesis in various models, suggesting a tumor suppressor function for caspase-2 [173]. Finding an inhibitory ubiquitylation of caspase-2 could help developing therapeutic tools for its tumor suppressor function.

On the other hand, caspase-2 has been implicated in neurodegenerative diseases such as Alzheimer’s Disease. A recent study showed that caspase-2 cleavage of Tau contributes to impairment of memory function in Alzheimer’s Disease [169]. Interestingly, Dronc-dependent Tau cleavage was also shown in a study that links circadian clock and Alzheimer’s Disease [345]. It will be compelling to investigate if K78 ubiquitylation of Dronc has an inhibitory function toward cleavage of Tau. Tau expression could induce a rough eye phenotype due to degeneration of photoreceptor neurons of *Drosophila* [346]. Furthermore, *shaggy* expression can enhance *tau*-induced rough eye phenotype through phosphorylating Tau which contributes to its toxicity [347]. An isoform of Shaggy, Shaggy46 was shown to be activated by caspase cleavage in SOP development [189]. It is not clear whether Shaggy46 could also phosphorylate Tau. However, it is intriguing to investigate whether Tau phosphorylation could also be
regulated through cleavage of Shaggy or Shaggy46 by caspases and contribute to toxicity of Tau in *Drosophila*. In this direction, examining whether Flag-Dronc^{K78R} could enhance Shaggy-induced Tau toxicity in *Drosophila* could be interesting. If Dronc contributes to Tau toxicity through cleavage of Tau or Shaggy, generation of a ubiquitin-mimetic Dronc to rescue Tau-dependent neurodegeneration will be intriguing to study.

A study questioning effects of apoptosis on aging discovered that caspase-2-deficient mice had decreased life span and increased aging-related traits [348]. Further studies identified that caspase-2 plays a role in responding oxidative stress, regulation of mitochondrial function and metabolic pathways, which can be possible mechanisms of how caspase-2 regulates aging [349]. If caspase-2 activity is negatively regulated by an inhibitory ubiquitylation similar to Dronc, it would be interesting to examine a non-ubiquitable caspase-2 in terms of aging. Similar to caspase-2, *dronc* null mutant flies also die in a few days after eclosion [61]. Moreover, another study showed that aging flies have increased levels of activated Dronc [345]. Interestingly, paraquat (a superoxide radical generator) injection into mice caused activation of caspase-2 and caspase-2 deficient mice displayed upregulation of JNK signaling [349]. A recent study from our lab also linked oxidative stress, JNK signaling and Dronc in AiP model [182]. It will be interesting to investigate whether a more active Dronc such as Flag-Dronc^{K78R} could have any impact on fly life span through influencing oxidative stress response, mitochondrial functions, metabolic pathways or other mechanisms yet to be discovered.

Caspase-1 is an important regulator of immune responses in mammals. Upon infectious stimuli, caspase-1 is activated after forming a protein complex called
inflammasome with NOD-like receptor proteins (such as NLRP3) through interactions between their CARD domains [173, 174]. Inflammasome is required for cleavage and maturation of cytokines IL-1β and IL-18 for immune responses [173, 174]. Inflammasome assembly is similar to the apoptosome assembly because in both cases, caspases and scaffolding proteins interact through their CARD domains with induced-proximity [173, 174]. Therefore, it is possible that the inflammasome formation may be regulated by ubiquitylation in CARD of caspase-1. In fact, caspase-1 CARD domain carries 8 lysine residues. Previous studies showed conflicting results on caspase-1 activation through ubiquitylation [350, 351]. One study suggested cIAP-1 and cIAP-2 conjugate K63 poly-ubiquitin chains on caspase-1, which promotes caspase-1 activation; whereas another study suggested combined depletion of XIAP, cIAP-1 and cIAP-2 leads to caspase-1 activation [350, 351]. None of these studies examined site of ubiquitylation and its mechanism in detail. It will be interesting to see if CARD domain of caspase-1 can be ubiquitylated and whether this potential ubiquitylation or reported K63 poly-ubiquitylation has any effect on inflammasome assembly.

As exampled above, K78 mono-ubiquitylation could open doors to new research revenues in the apoptosis field. Testing function of this ubiquitylation in the context of Dronc’s activation and its mammalian counterpart by extension could allow us to make discoveries which will benefit our understanding of the apoptotic machinery and caspase activation. Studying this ubiquitylation and its relationship to Dronc’s catalytic activity in the context of its non-apoptotic functions may also result in identifying new regulatory
mechanisms that could help us understand roles of caspases in apoptosis-independent cellular pathways.
APPENDIX

The initiator caspase Dronc is subject of enhanced autophagy upon proteasome impairment in Drosophila

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Abstract

A major function of ubiquitylation is to deliver target proteins to the proteasome for degradation. In the apoptotic pathway in *Drosophila*, the inhibitor of apoptosis protein 1 (Diap1) regulates the activity of the initiator caspase Dronc (Caspase-9 ortholog) by ubiquitylation, supposedly targeting Dronc for degradation by the proteasome. Using a genetic approach to analyze proteasome function in epithelial cells *in vivo*, we show that Dronc is not subject of proteasome-mediated degradation, contrary to the expectation. Likewise, an alternative catabolic pathway, autophagy, does not mediate degradation of Dronc either. However, combined impairment of the proteasome and autophagy triggers accumulation of Dronc protein levels suggesting that autophagy compensates for the loss of the proteasome with respect to Dronc turnover. Consistently, we show that loss of the proteasome enhances endogenous autophagy in epithelial cells. We propose that enhanced autophagy degrades Dronc if proteasome function is impaired.

Introduction

There are two major catabolic pathways in eukaryotic cells that degrade the bulk of cellular proteins, the ubiquitin-proteasome system (UPS) and macro-autophagy, usually referred to as autophagy [295, 352-354]. In the UPS, poly-ubiquitylated proteins are delivered to the 26S proteasome for degradation. The 26S proteasome consists of a 20S catalytic core, flanked by two 19S regulatory complexes [355, 356]. The 20S catalytic core is composed of a total of 28 α- and β-type subunits which are organized in a barrel with four stacked rings. The outer two rings are formed by seven α-type subunits
each, the two inner rings by seven β-type subunits each [355]. All α- and β-type subunits are needed for structural integrity of the proteasome [357]. Three β-type subunits, β1, β2 and β5, have proteolytic activity [356]. The 19S regulatory particle is composed of at least nineteen subunits involved in recognition of ubiquitin-conjugated substrates, ATP hydrolysis, de-ubiquitination, protein unfolding and feeding of the substrates into the 20S catalytic core for degradation [355]. Genetic studies in yeast and Drosophila have revealed that mutations in many subunits of the 20S core and the 19S regulatory domains impair proteasome function [358, 359]. Genetic analysis of proteasome function is also of clinical importance as proteasome inhibition may be used as potential anti-tumor strategy, especially for treatment of multiple myeloma [360-363].

Autophagy is characterized by the formation of double-membrane vesicles termed autophagosomes [364, 365]. During autophagosome maturation, cytosolic proteins and entire organelles are trapped and delivered to the lysosome for degradation. Two ubiquitin-like conjugation pathways (Atg8/LC3 and Atg12) are active during maturation of autophagosomes [364, 365]. Atg7 is an E1-activating enzyme involved in both conjugation pathways [364] and essential for autophagy [366]. The incorporation of Atg8 fusion proteins (for example with GFP or mCherry) into autophagosomes is often used as a marker for autophagosomes [367] and autophagic flux [368].

While it was initially assumed that the UPS system and autophagy are independent of each other, recent evidence has suggested that there is crosstalk and feedback between the two [352, 369-377] (reviewed by [353, 378, 379]). This is mostly due to the observation that autophagy can also degrade ubiquitylated proteins [380-384].
Mechanistically, p62, an adaptor protein with an ubiquitin-binding domain and a LC3 interacting region (LIR), links poly-ubiquitylated proteins to LC3/Atg8 at the autophagosome [380]. Loss of autophagy can lead to the formation of protein aggregates composed of poly-ubiquitylated proteins and p62. These protein aggregates are frequently associated with neurodegenerative diseases in humans [385-387].

Apoptosis is the major form of cell death and evolutionarily well conserved from flies to humans [283, 388]. Caspases are highly specific Cys-proteases and are the main effectors of apoptosis. They are produced as inactive precursor zymogens that are activated either through incorporation into large protein complexes such as the apoptosome (initiator caspases) or by proteolytic processing (effector caspases) [173, 286]. After activation in the apoptosome, initiator caspases such as Caspase-9 and its Drosophila ortholog Dronc cleave and activate effector caspases such as Caspase-3 and its Drosophila ortholog DrICE [4, 283].

The activity of caspases is controlled at multiple levels. In addition to zymogen production, apoptosome-mediated activation of Caspase-9/Dronc and proteolytic processing of Caspase-3/DrICE, caspases are also inhibited by inhibitor of apoptosis proteins (IAPs), most notably XIAP in mammals and Diap1 in Drosophila [4]. IAPs carry a RING domain which has E3 ubiquitin ligase activity [291]. In Drosophila, because the RING domain of Diap1 ubiquitylates Dronc [78-80, 389], it is commonly assumed that this ubiquitylation targets the caspase for proteasome-mediated degradation [79, 80]. However, in vivo this has not been observed. On the contrary, we have shown that loss or gain of Diap1 does not affect the protein levels of Dronc in surviving cells.
[89]. A similar observation has been reported for DrICE [389]. Furthermore, a mouse mutant deleting the RING domain of XIAP does not significantly affect caspase protein levels [146]. Therefore, it is currently unclear how the protein levels of Dronc are controlled in living cells to avoid deleterious accumulation and potentially auto-processing of these dangerous proteins.

Traditionally, in *Drosophila* the function of autophagy has been studied mainly in large non-epithelial cells such as the fat body, midgut and salivary glands, because these tissues require autophagy for turnover during metamorphosis [364]. A role of autophagy in epithelial cells such as larval imaginal discs has been less explored. Here, we report that in epithelial cells the protein levels of Dronc are unaffected by proteasome impairment. Similarly, loss of autophagy by itself does not affect Dronc protein levels. However, simultaneous impairment of the proteasome and autophagy causes accumulation of Dronc suggesting that autophagy can compensate for the loss of the proteasome with respect to Dronc turnover. Consistently, autophagy is enhanced in proteasome mutants in epithelial cells. In summary, these data identify Dronc as a common substrate for both the proteasome and autophagy.

**Results**

**Accumulation of polyubiquitylated proteins is a convenient marker for proteasome dysfunction**

Ubiquitylation of Dronc by Diap1 has previously been observed *in vitro* [78, 80]. To directly test if ubiquitylated Dronc is degraded by the proteasome, we analyzed Dronc
protein levels in two mutants affecting the proteasome. The first mutant, \textit{prosβ2}, also known as \textit{DTS7} in \textit{Drosophila} [390], affects the β2 subunit of the 20S catalytic core of the proteasome. The Prosβ2 subunit provides both structural integrity to the proteasome and proteolytic activity [357]. The second mutant affects the \textit{Mov34} gene (also known as p39B) which encodes a subunit in the 19S regulatory complex, corresponding to Rpn8 in yeast and S12 in the human regulatory complex [391]. Both mutants, \textit{prosβ2}^{EP3067} and \textit{Mov34}^{k08003}, are caused by P-element insertions in the first exon which likely disrupt the transcripts. Both proteasome mutants behave identically in our assays (see below). Because these proteasome mutants are homozygous lethal, we induced mutant clones using the \textit{ey-Flp/FRT} system [392, 393]. Not unexpectedly, mutant clones affecting the proteasome are very small and are difficult to identify using negative selection with GFP (Figure A1A-C). However, we were able to positively mark and identify mutant clones using antibodies that recognize ubiquitin or ubiquitin-conjugated proteins. Antibodies raised against ubiquitin display increased immunoreactivity in \textit{prosβ2} mutant clones (Figure A1A’). There is a perfect match between the GFP-negative areas which mark the \textit{prosβ2} mutant clones, and increased abundance of ubiquitin labeling (Figure A1A’’). Because poly-ubiquitin-conjugated proteins are usually subject to proteasome-mediated degradation, we tested whether ubiquitin-conjugated proteins account for the accumulation of ubiquitin in \textit{prosβ2} mutants. The FK1 and FK2 antibodies specifically recognize ubiquitin-conjugated proteins, but not unconjugated ubiquitin (FK1 labels poly-ubiquitylated conjugates, FK2 labels mono- and poly-ubiquitylated proteins)
Figure A1. *prosβ2* mutant cells accumulate ubiquitin-conjugated proteins.

Shown are high magnification images (100X) of the posterior compartment of *prosβ2* mosaic eye imaginal discs labeled for ubiquitin (A), FK2 (B) and FK1(C). The FK2 and FK1 antibodies detect ubiquitin-conjugated proteins, but not free unconjugated ubiquitin. The left panels indicate the positions of the proteasome mutant clones by absence of GFP, the middle panels show the experiment (in magenta), and the right panels are the merged images of left and middle panels. White arrows mark a few clones as examples. In this and all other figures, anterior is to the left. Similar data were obtained for *Mov34* mosaic discs (Figure A2). Wild-type mosaic control discs do not show accumulation of ubiquitin (Figure A2). Genotype: *ey-FLP; prosβ2*<sup>EP3067</sup> *FRT80 / ubi-GFP FRT80*.
Figure A2. *Mov34* mutants accumulate ubiquitin-conjugated proteins.

Shown are high magnification images (100X) of the posterior compartment of *Mov34* mosaic eye imaginal discs labeled for FK2 (A’, A’’) and FK1(B’, B’’). The FK2 and FK1 antibodies detect ubiquitin-conjugated proteins, but not free unconjugated ubiquitin. The left panels indicate the positions of the proteasome mutant clones by absence of GFP (A, B), the middle panels show the experiment (in magenta), and the right panels are the merged images of left and middle panels. White arrows mark a few clones as examples. Genotype: *ey-FLP; FRT42D Mov34<sup>k08003</sup> / FRT42D P[ubi-GFP]*.
Figure A3. Wild-type control mosaic discs do not accumulate ubiquitin and ubiquitin-conjugated proteins.

Shown are wild-type (wt) mosaic eye imaginal control discs (wt clones in wt background) labeled for ubiquitin (A, A’), FK1 (B, B’) and FK2 (C, C’). Control clones are marked by the absence of GFP. Ubiquitin and ubiquitin-conjugates do not accumulate in control clones. Genotype: ey-FLP; FRT42D P[ubi-GFP] / FRT42D.
[303, 394]. As shown in Figure A1B, C, the immunoreactivity of FK1 and FK2 antibodies increases in \(pros\beta2\) mutant clones. We also find increased FK1 and FK2 labeling in \(Mov34\) mutant clones (Figure A2). In wild-type (wt) control mosaics (wt clones in wt background), an accumulation of ubiquitin and conjugated ubiquitin (FK1/2) is not observed (Figure A3) suggesting that ubiquitin-conjugated proteins specifically accumulate in proteasome-deficient cells \textit{in vivo}, consistent with the expectation.

**Mutations in proteasome subunits cause cell death**

Based on the results presented above, we used FK2 labeling to positively mark and identify proteasome mutant clones. First, we examined the consequence of proteasome dysfunction for the survival of the affected cells. The rationale of this experiment was that in clinical applications, proteasome inhibitors are used to induce apoptosis in cancer patients including multiple myeloma [361, 363]. Consistently, pharmacological inhibition of the proteasome by Bortezomib in whole flies resulted in DrICE cleavage after 4 days of treatment [377]. This treatment correlated with a strong reduction of longevity of the affected animals. In contrast, tissue-specific inhibition of the proteasome in fat body cells by RNAi did not confirm such an apoptotic response [395].

Because RNAi is known to cause partial loss-of-function phenotypes, we tested the strong \(pros\beta2\) mutant allele in mosaic eye-antennal imaginal discs for apoptosis induction. Indeed, using cleaved caspase-3 antibody labelings, we observed activated caspases in proteasome-deficient clones (Figure A3). However, not all mutant clones contain active caspases. Nevertheless, whether apoptotic or not, all clones are very small indicating that the mutant cells do not grow very well.
Figure A4. Mutations in proteasome subunits result in cell death.

Shown are high magnification images (100X) of the posterior compartment of prosβ2 mosaic eye imaginal discs labeled for cleaved caspase-3 (Csp3) and FK2 to identify prosβ2 mutant clones. Csp3 labeling is increased in some, but not all prosβ2 mutant clones (red in A’ and A”). Genotype: ey-FLP; prosβ2EP3067 FRT80 / ubi-GFP FRT80.
Mutant clones of proteasome subunits accumulate Diap1, but do not affect Dronc protein levels

Because Diap1 can ubiquitylate Dronc \textit{in vitro}, [78, 80] it was predicted that Dronc is subject to proteasome-mediated degradation in surviving cells [79, 80]. It follows that in surviving cells the protein levels of Dronc would accumulate in proteasome mutants. We tested this prediction by analyzing \textit{prosβ2} and \textit{Mov34} mutant clones located in the posterior eye imaginal disc of third instar larvae. We chose the posterior eye imaginal disc in this analysis because under normal conditions, there is no developmental apoptosis in this tissue, thus avoiding complications with physiological apoptosis. Surprisingly, Dronc protein levels are not detectably altered in \textit{prosβ2} and \textit{Mov34} mutant clones (Figure A5A, B). We also found the same result using a different \textit{prosβ2} mutant allele, the commonly used \textit{DTS7} allele (Figure A6), although this allele did not cause accumulation of poly-ubiquitylated proteins (FK1 antibody labeling) suggesting that it is a weaker allele as the one used in Figure A5A. The specificity of the anti-Dronc antibody was previously established in \textit{dronc} mosaic eye and wing imaginal discs, and the antibody can also detect accumulated Dronc protein [89].

However, to further confirm the lack of Dronc accumulation in proteasome mutants, we analyzed the protein levels of a heterologously expressed Flag-tagged Dronc [98] in proteasome mosaic eye discs. Using the Flag antibody as a tool to monitor Dronc levels, we also did not detect an accumulation of Flag-Dronc in \textit{Mov34} mutant cells (Figure A7).
Figure A5. Diap1, but not Dronc, accumulate in proteasome mutant clones.

Shown are high magnification images (100X) of the posterior compartment of *prosβ2* (A,C) and *Mov34* (B,D) mosaic eye imaginal discs labeled for Dronc (A,B) and Diap1 (C,D). FK2 labeling was used to identify mutant clones. The left panels indicate the positions of the proteasome mutant clones by absence of GFP. In the middle panels, the
proteasome mutant clones are positively marked by FK2 labeling (in magenta). The right panels show the Drone (A”, B”) and Diap1 labelings (C”, D”) in red. White arrows mark a few clones as examples.

Genotype in (A) and (C): ey-FLP; prosβ2EP3067 FRT80 / P[ubi-GFP] FRT80. Genotype in (B) and (D): ey-FLP; FRT42D Mov34k08003 / FRT42D P[ubi-GFP].
Figure A6. Mutant clones of the DTS7 allele of prosβ2 do not accumulate Dronc.

Mosaic prosβ2<sup>DTS7</sup> eye imaginal discs labeled for Dronc (A’, A”) and FK1 (A”’, A”’’). Mutant clones are marked by the absence of GFP (A). Dronc does not accumulate in mutant clones. However, FK1 labeling is also not enhanced suggesting that DTS7 in homozygous clones is a weak allele. The cross was performed at 25°C. Genotype: ey-FLP; prosβ2<sup>DTS7</sup> FRT80 / P[ubi-GFP] FRT80.
Shown is the posterior compartment of a Mov34 mosaic eye imaginal disc expressing Flag-dronc by the da-Gal4 driver. Mov34 clones are marked by the absence of GFP (A, A’). Heterologous Flag-Dronc protein levels are detected using an anti-Flag antibody (A, A’’). White arrows highlight a few select Mov34 clones as examples. Flag-Dronc does not accumulate in Mov34 clones. Genotype: ey-FLP; FRT42D Mov34\textsuperscript{k08003} / FRT42D P[ubi-GFP]; da-Gal4 UAS-Flag-dronc.
As a positive control, we analyzed Diap1 protein levels in proteasome mutants. Diap1 degradation in dying cells has been demonstrated in vivo [82, 84, 85, 273]. Consistently, we observe accumulation of Diap1 in prosβ2 and Mov34 mutant clones (Figure A5C, D). Thus, our assay can detect accumulating proteins in vivo.

Because there is no or only very little apoptosis occurring at the investigated stage of the mosaic discs (obtained from late third instar larvae), these data suggest that the protein levels of Diap1 in living cells are regulated by the UPS system. In contrast, protein levels of Dronc in living cells are either not regulated by the UPS system, or there are compensatory mechanisms operating that turn over Dronc upon proteasome dysfunction.

Loss of the autophagy gene Atg7 does not affect Dronc protein levels

In addition to the UPS, autophagy is a cellular catabolic process known to degrade proteins [352, 354, 365]. We considered the possibility that autophagy may regulate the protein levels of Dronc. As described in the Introduction, the autophagy gene Atg7 encodes the E1-activating enzyme for the two ubiquitin-like conjugation systems and is an essential gene for autophagy [364, 366]. Therefore, because proteasomal dysfunction does not affect the protein levels of Dronc in living epithelial cells, we tested the possibility that autophagy may control it. For that purpose, we down-regulated Atg7 function by RNAi or inactivated Atg7 in mutant clones. However, similar to proteasomal dysfunction, impaired autophagy does not affect Dronc protein levels in mosaic eye-antennal imaginal discs (Figure A8A, B).
Figure A8. Loss of Atg7 alone does not affect Dronc protein levels.

Dronc labeling of Atg7 mosaic eye imaginal discs. Atg7 was either down-regulated by RNAi (A) or genetically inactivated in mutant clones (B). Atg7-deficient clones are marked by absence of GFP. White arrows highlight one clone in each panel as example. Dronc protein levels are unaltered in Atg7-deficient clones.

Genotypes: (A) yw hsFLP; tub>GFP>Gal4 / UAS-Atg7^{12-3} (> denotes FRT). (B) yw eyFLP; FRT42D Atg7^{d14} / FRT42D P[ubi-GFP].
Simultaneous inactivation of both the proteasome and autophagy triggers accumulation of Dronc protein

We examined the possibility that the protein levels of Dronc are coordinately regulated by both catabolic pathways. To address this question, we inactivated autophagy by ATG7 RNAi in prosβ2 mutant clones using the MARCM method [319]. Indeed, simultaneous impairment of both the proteasome and autophagy causes strong accumulation of Dronc protein in eye-antennal imaginal discs (Figure A9A). Similar observations were also made in wing imaginal discs, another epithelial tissue (Figure A9B). The accumulation of Dronc in proteasome/autophagy double-deficient clones is not due to a transcriptional upregulation of dronc transcripts (Figure A10). Together, these data suggest that Dronc protein levels are coordinately regulated by both the proteasome and autophagy.

Loss of proteasome function enhances autophagy in epithelial cells

To explain the synergistic control of Dronc protein levels by the UPS system and autophagy, we considered two scenarios. First, it is possible that both catabolic processes degrade Dronc independently of each other and if one process is lost, the other one can still maintain Dronc at normal protein levels. In the second scenario, we considered that, because the UPS system and autophagy are mechanistically linked, impairment of the UPS can enhance autophagy which is often referred to as compensatory autophagy [352, 369-377] (reviewed by [353, 378, 379]). For example, in Drosophila, compensatory autophagy after proteasome impairment has been reported in neurons, in fat body cells
Figure A9. Dronc accumulates in clones simultaneously mutant for the proteasome and autophagy.

Mosaic eye (A) and wing (B) imaginal discs doubly deficient for the proteasome and autophagy labeled for Dronc (red). Atg7 knockdown was induced by RNAi in prosβ2 mutant clones using the MARCM method. Clones are marked by GFP. Dronc accumulates in double deficient clones. The cross was performed at 25°C. Genotype: hs-FLP UAS-GFP tub-Gal4; UAS-Atg7^{12-3}/+; prosβ2^{DTS7} FRT80B/FRT80B tub-Gal80 FRT80B.
Figure A10. *dronc* transcripts do not accumulate in proteasome and autophagy-deficient animals.

Late L3 larvae of control (*w*1118) and experimental genotype (*w*; *UAS-Atg7RNAi*; *da-Gal4*/*DTS7*) were shifted to 29°C until eclosion of adult flies. mRNA was extracted from 1-3 days old flies. Extracted RNA was subjected to qPCR for *dronc* and *tubulin* (as control). *dronc* transcripts are not significantly increased in proteasome and autophagy-deficient animals. This experiment was performed twice. The student t-test was used. Error bars are SD. ns – not significant.
and in adult flies [372, 377, 395]. To examine this scenario in epithelial cells, we monitored autophagy using a tandem fusion protein GFP-mCherry-Atg8 as reporter for autophagic flux [368]. This reporter is incorporated into autophagosomes which mature into autolysosomes. In autolysosomes, fluorescence of the GFP moiety of the reporter is quenched, while mCherry signals persist. Therefore, this reporter is suited to monitor autophagic flux from autophagosomes into autolysosomes. Indeed, mCherry signals, but not GFP, strongly accumulate in *Mov34* mutant clones (Figure 6) suggesting that autophagic flux is induced in response to proteasome impairment.

**Discussion**

In this paper, we examined the consequences of impaired proteasome function on apoptosis and components of the apoptotic pathway in epithelial cells. We observed that proteasome dysfunction triggers apoptosis in some, but not all, mutant clones (Figure A4). We also found that protein levels of Diap1, but not Dronc, accumulate in proteasome-deficient cells (Figure A5). Dronc also does not accumulate in autophagy-deficient cells in which the E1 encoding gene Atg7 was mutant or down-regulated by RNAi (Figure A8). However, simultaneous inactivation of the proteasome and Atg7 resulted in strong accumulation of Dronc (Figure A9). In addition, autophagy is strongly enhanced in proteasome mutants (Figure A11).

Our data indicate that Dronc is a shared substrate for degradation by both the UPS and autophagy. There are not many substrates known which are common to both the UPS and autophagy. α-synuclein is one substrate and mutant forms of this protein appear to
Figure A11. Impaired proteasome function induces autophagic flux.

Mov34 mosaic eye imaginal discs expressing GFP-mCherry-Atg8a as marker for autophagic flux. Mov34 mutant cells were identified by FK2 labeling (blue in a; gray in a’). White arrows mark representative Mov34 mutant cells as examples. Although there is little to no GFP labeling in Mov34 mutant cell clones (a”), all clones contain increased mCherry labeling (a”’) suggesting that autophagic flux is enhanced in proteasome-deficient cells. Genotype: ey-FLP;

FRT42D Mov34k08003 /FRT42D; patg8a4GFP-mCherry-Atg8a
poison both the UPS and autophagy causing Parkinson’s disease [396-398]. Another shared substrate is \( \text{IkB}\alpha \), the inhibitor of the transcription factor NF-\( \kappa \)B.[399] \( \text{IkB}\alpha \) appears to be degraded by the UPS and autophagy in different cellular compartments with different rates [399]. In addition to these specific shared substrates, misfolded proteins are common substrates for both the UPS and autophagy [379, 400]. Whether this relates to Dronc, is currently unknown.

It is unclear what distinguishes Dronc from Diap1 with respect to proteasome-mediated degradation and enhanced autophagy. The alternative question would be why Diap1 is not degraded by enhanced autophagy in proteasome-deficient cells. There may be specific ubiquitylation marks or other post-translational modifications that distinguish between these possibilities. Additional work is necessary to answer these questions in the future.

Given the accumulation of the anti-apoptotic protein Diap1 in proteasome-deficient cells, it is somewhat surprising that some of them undergo apoptosis. However, many other proteins likely also accumulate in proteasome-deficient cells which combined will tilt the fate of the affected cells to either survival or death depending on relative ratios. This consideration may explain why some cells are apoptotic and others are not. Another possibility is that the non-apoptotic cells in mutant clones will die later or are already dead. Consistent with the latter notion, we have previously shown that dead cells lack protein and DNA [401], hence the cleaved caspase-3 antibody would not label these cells. Importantly also, even though not all proteasome-deficient cells may be apoptotic at the time of investigation, they do not grow very well and remain small. These
considerations are important for the potential clinical use of proteasome inhibitors for treatment of cancer.

Genetic analysis of proteasome function

This is the first report in which a clonal analysis of proteasome mutants was performed. Usually, dominant temperature sensitive (DTS) alleles of proteasome subunits (DTS5, DTS7, etc.), RNAi or pharmacological inhibition have been used to study proteasome function [358, 372, 377, 390, 395, 402]. In other approaches, whole embryos mutant for proteasome subunits were characterized for defects in dendrite pruning in sensory neurons in Drosophila [193, 403, 404]. However, a specific analysis characterizing recessive alleles for defects in proteasome activity has not been reported. The reasons for this omission are obvious. Mutant animals are homozygous lethal and mutant clones in otherwise heterozygous animals are very small and difficult to identify. We found that cells mutant for proteasome function accumulate ubiquitin-conjugated proteins (Figure A1), consistent with the expectation. We used markers detecting ubiquitin-conjugated proteins to positively identify mutant clones. That enabled us to identify Diap1 as substrate of the proteasome, whereas control of Dronc protein levels appears to be independent of the proteasome (Figure A5). The proteasome alleles used in this study and our approach to identify mutant clones will be of general use for future analysis of proteasome function.

Methods
Fly stocks: $pros\beta^2_{EP3067}$ is a P element transposon insertion in the first exon at base pair 63 [405]. $Mov34_{408003}$ is a P element transposon insertion in the first exon at base pair 263 [403, 404]. Both insertions disrupt the transcripts. $pros\beta^2_{DTS7}$ encodes a dominant temperature sensitive allele of $pros\beta^2$; however, in this work, we used it in genetic mosaics using $FRT80B\ DTS7$, not applying a temperature-shift. $Atg7^{dl14}$ is a mutant allele as described [366]. $UAS-ATG7^{dl2-3}$ and $UAS-ATG7^{dlR}$ (VDRC TID45561) are RNAi lines that target $ATG7$. $UAS-Flag-dronc$ encodes a Dronc protein with an N-terminal Flag-tag [98]. It was expressed using $daughterless-Gal4$ ($da-Gal4$) in $Mov34$ mosaic background (Figure A7). A transgene encoding the tandem protein GFP-mCherry-ATG8a was generated and used to determine autophagic flux [368].

Generation of mutant clones: Mutant clones of $pros\beta^2$, $Mov34$ and $Atg7$ were induced in eye-antennal imaginal discs using the $FLP/FRT$-induced mitotic recombination system using $ey-FLP$ [392, 393]. For this purpose, mutant alleles of $pros\beta^2$ and $Mov34$ were recombined on $FRT80B$ and $FRT42D$ bearing chromosomes, respectively. $FRT42D\ Atg7^{dl14}$ was used as described [366]. To generate wild-type control mosaics (Figure A3), $ey-Flp$; $FRT42D\ P[ubi-GFP]$ was used. Clones are marked by loss of GFP. To induce $Atg7$ RNAi in $pros\beta^2$ mutant clones, the MARCM method was used [319].

Immunohistochemistry: Eye-antennal imaginal discs from third instar larvae were dissected using standard protocols [322] and labeled with antibodies raised against the following antigens: Ubiquitin (Sigma); FK1 and FK2 (Biomol); cleaved Caspase-3 (Cell Signaling Technology); Dronc (kind gift of Pascal Meier), Diap1 (a kind gift of Hermann
Steller and Hyung Don Ryoo) and Flag (Sigma Aldrich). Cy3- and Cy-5 fluorescently-conjugated secondary antibodies are obtained from Jackson ImmunoResearch and were used at dilutions of 1:400. In each experiment, multiple clones in 10-20 eye imaginal discs were analyzed, unless otherwise noted. Images were captured using Olympus Optical FV500 or Zeiss LSM700 confocal microscopes.

**Generation of the GFP-mCherry-Atg8a tandem reporter:** A transgene encoding the tandem protein GFP-mCherry-Atg8a was generated and used to determine autophagic flux. A region 2-kb upstream of Atg8a (CG32672) was inserted upstream of GFP-mCherry-Atg8a in the pCaSpeR4 Drosophila transformation vector, as was previously described for similar GFP-Atg8a and mCherry-Atg8a reporter lines [406, 407]. The resulting plasmid pCaSpeR4-promoter GFPmCherry-Atg8a was used to generate transgenic Drosophila lines using standard procedures.

**qPCR of dronc transcripts:** Late 3rd larval stage (L3) larvae of control (w^{118}) and experimental genotype (w; UAS-Atg7^RNAi; da-Gal4/DTS7) were shifted to 29 °C until eclosion of adult flies. mRNA was extracted from 1 to 3 days old flies using RNAeasy (Qiagen, Hilden, Germany) and quantitative polymerase chain reaction (qPCR) was performed with the following dronc-specific primers: Nedd2-like caspase forward primer (NcF) 5’-CTCGCTAAACGAACGGAGAAC-3’ and Nedd2-like caspase reverse primer (NcR) 5’-CAACGACACCCACATAAGGG-3’, as described [408]. Tubulin was used for normalization.

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