Discovery of a Novel Signaling Circuit Coordinating *Drosophila* Metabolic Status and Apoptosis

by

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Department of Pharmacology and Cancer Biology
Duke University

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Xiao-Fan Wang

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2011
ABSTRACT

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Abstract

Apoptosis is a conserved mode of cell death executed by a group of proteases named caspases, which collectively ensure tissue homeostasis in multicellular organisms by triggering a program of cellular “suicide” in response to developmental cues or cellular damage.

Accumulating evidence suggests that cellular metabolism impinges directly upon the decision to initiate cell death. Several links between apoptosis and metabolism have been biochemically characterized. Using Xenopus oocyte extracts, our laboratory previously discovered that caspase-2 is suppressed by NADPH metabolism through an inhibitory phosphorylation at S164. However, the physiological relevance of these findings has not been investigated at the whole organism level. Studies presented in this dissertation utilize both Schneider’s Drosophila S2 (S2) cells and transgenic animals to untangle the influence of metabolic status on fly apoptosis.

We first demonstrate a novel link between Drosophila apoptosis and metabolism by showing that cellular NADPH levels modulate the fly initiator caspase Dronc through its phosphorylation at S130. Biochemically and genetically blocking NADPH production removed this inhibitory phosphorylation, resulting in the activation of Dronc and the subsequent apoptotic cascade in cultured S2 cells and specific neuronal cells in transgenic animals. Similarly, non-phosphorylatable Dronc was found to be more potent than wild-type in triggering neuronal apoptosis. Moreover, upregulation of NADPH prevented Dronc-mediated apoptosis upon abrogation of Drosophila Inhibitor of
Apoptosis (IAP) protein 1 (DIAP1) by double-stranded RNA (dsRNA) or cycloheximide (CHX) treatment, revealing a novel mechanism of DIAP1-independent apoptotic regulation in *Drosophila*. Mechanistically, the CaMKII-mediated phosphorylation of Dronc hindered its activation, but not its catalytic activity. As NADPH levels have been implicated in the regulation of oocyte death, we demonstrate here that a conserved regulatory circuit also coordinates somatic apoptosis and NADPH levels in *Drosophila*.

Given the regulatory role of NADPH in the activation of Dronc in *Drosophila* and caspase-2 in vertebrates, we then attempted to further elucidate the underlying signaling pathways. By tracking the catabolic fate of NADPH, we revealed that fatty acid synthase (FASN) activity was required for the metabolic suppression of Dronc, as both the chemical inhibitor orlistat and FASN dsRNA abrogated NADPH-mediated protection against CHX-induced apoptosis in S2 cells. Interestingly, it has been previously demonstrated that blocking FASN induces cell death in numerous cancers, including ovarian cancer; however, the mechanism is still obscure. As our results predict that suppression of FASN activity may prevent the inhibitory phosphorylation of Dronc and caspase 2 (at S130 and S164 respectively), we examined the contribution of caspase-2 to cell death induced by orlistat using ovarian cancer cells. Indeed, caspase-2 S164 was dephosphorylated upon orlistat treatment, initiating the cleavage and activation of caspase-2 and its downstream target, Bid. Knockdown of caspase-2 significantly alleviated orlistat-induced cell death, further illustrating its involvement.
Lastly, we developed an assay based on bimolecular fluorescence complementation (BiFC) to monitor the oligomerization of Dronc in S2 cells, a crucial step in its activation. The sensitivity of this assay has been validated with several apoptotic stimuli. A future whole-genome screen employing this assay is planned to provide new insights into this complex apoptotic regulatory network by unbiasedly identifying novel apoptotic regulators.
Dedication

To my parents and Shaoyu,

for their unconditional love and support
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List of Abbreviations

420 OVCA420
432 OVCA432
AIF Apoptosis-inducing factor
Apaf-1 Apoptotic protease activating factor-1
BiFC Bimolecular fluorescence complementation
BIR Baculoviral IAP Repeat
BSA Bovine serum albumin
CaMK Calcium/calmodulin-dependent protein kinase
CARD Caspase recruitment domain
Caspase Cysteinyl aspartate-specific protease
CDK Cyclin dependent kinase
CHX Cycloheximide
CK1 Casein kinase 1
Crm A Cytokine Response modifier A
Dark Drosophila Ark/dApaf-1/Hac-1
DD Death domain
DED Death effector domain
DHEA Dehydroepiandrosterone
DIAP1 Drosophila inhibitor of apoptosis protein 1
DISC Death inducing signaling complex
DMSO Dimethyl sulfoxide
drICE Drosophila ICE
Dronc Nedd2-like caspase/Nc
dsRNA Double-stranded RNA
ER Endoplasmic reticulum
EX Totally expended
<table>
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<th>Description</th>
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<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IBM</td>
<td>IAP binding motif</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1 beta-converting enzyme</td>
</tr>
<tr>
<td>Malate</td>
<td>Dimethyl-L-malate</td>
</tr>
<tr>
<td>Men</td>
<td>Malic enzyme</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>PI-3K/Rac1/p21-activated kinase</td>
</tr>
<tr>
<td>PEX</td>
<td>Partially expanded</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
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<tr>
<td>PIDD</td>
<td>p53-induced protein with a death domain</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PPI</td>
<td>Protein phosphatase-1</td>
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<td>RAIDD</td>
<td>RIP-associated ICH-1/CED-3 homologous protein with a death domain</td>
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<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>ROS</td>
<td>Reactive oxidative species</td>
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<td>rpr</td>
<td>reaper</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>S2</td>
<td>Schneider’s <em>Drosophila</em> S2</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SUMO</td>
<td>Small ubiquitin-related modifier</td>
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<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>UEX</td>
<td>Unexpanded</td>
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<td>UPS</td>
<td>Ubiquitin proteasome pathway</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>v-FLIP</td>
<td>Viral FLICE-inhibitory protein</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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<tr>
<td>zVAD</td>
<td>z-VAD-fluoromethylketone</td>
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This has been such a journey of surprise. Back when I just began my graduate school in the summer of 2006, I cannot have imagined that one day I will be able to tell the sex of a fly without using a microscope, and talk to my parents about how many “virgins” I collected tonight. I have been very fortunate to have many people to thank for their guidance and support during my training as an apprentice in the fly world.

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Chapter 1 Introduction

1.1 Apoptosis

Apoptosis refers to a form of genetically regulated cell death by which individual cells are sacrificed for the welfare of the whole organism. Before it was coined “apoptosis” by Kerr, Wyllie and Currie in 1972, many terms, including necrobiosis, shrinkage necrosis and chromatolysis, had been used to describe this manner of death characterized by reduced cell volume, condensed nuclear chromatin and formation of membrane-bound vesicles (known as “apoptotic bodies”) (Afford and Randhawa, 2000; Rich et al., 1999). Subsequent studies revealed additional features of apoptotic cell death at the molecular level, such as DNA fragmentation and the flip of phosphatidylserine from the inner to the outer plasma membrane leaflet, permitting quantitative assessment of apoptotic cell death (Duke et al., 1983; Martin et al., 1995; Wyllie, 1980). All of these morphological and biochemical characteristics distinguish apoptosis from other types of cell death and eventually became the gold standard of the field (Figure 1.1).

1.1.1 Physiological functions of apoptosis

Apoptosis plays an essential role in many physiological processes. During development, apoptosis is actively involved in embryo shaping and organogenesis, including proper formation of the extremities in embryos (Zou and Niswander, 1996). Genetic ablation of key apoptotic regulators, such as Apaf-1, cytochrome c, caspase-9, and caspase-3, causes hyperplasia in the central nervous system and perinatal death in
homozygous knockout mice, demonstrating the requirement of apoptosis in neuron development (Kumar, 2007).

Apoptosis also mediates physiological cell turnover to maintain homeostasis in mature tissues, including the removal of autoreactive cells in the immune system and malfunctioning or infected cells to prevent further damage (Barber, 2001; Bouillet et al., 1999). The role of aberrant apoptosis in numerous diseases further illustrates the importance of this surveillance system. For example, neurodegenerative diseases have been associated with excessive apoptotic cell death, whereas compromised apoptosis contributes to autoimmune diseases and oncogenesis (Bouillet et al., 1999; Ekshyyan and Aw, 2004; Hanahan and Weinberg, 2011). Thus, dissection of the apoptotic regulatory network not only enriches the knowledge of this biological phenomenon, but leads to new insights for the development of novel therapies.

1.1.2 Apoptotic signaling pathways

Apoptotic signaling pathways have been characterized in several model organisms including nematodes, flies and vertebrates. While the complexity of the apoptotic regulatory network has increased over the course of evolution, the main scheme is conserved across species (Kumar, 2007; Twomey and McCarthy, 2005). Here, the apoptotic signaling pathways in vertebrates is described to exemplify this principle; the commonality and distinct features of Drosophila apoptosis are reviewed in section 1.3.1.

At the center of the apoptotic machinery is a group of Cysteiny1 ASPartate-specific proteASEs, termed caspases, that use a cysteine nucleophile to cleave substrates
following an Asp residue (Thornberry and Lazebnik, 1998). In healthy cells, caspases are produced as inactive zymogens consisting of a prodomain, a large subunit, and a small subunit (Figure 1.2). Upon receiving a death stimulus, caspases are activated via proteolytic cleavage and/or oligomerization to mediate the cleavage of various cellular substrates, which in turn promotes well-defined morphological changes and cell death (Boatright and Salvesen, 2003; Danial and Korsmeyer, 2004; Fischer et al., 2003).

Based on their functional niche in the apoptotic signaling cascade, caspases can be further categorized into “apical/initiator” and “effector/executor” (Fuentes-Prior and Salvesen, 2004; Kumar, 2007). Initiator caspases, such as caspase-2, -8, and -9 in vertebrates and Dronc in Drosophila, can be recruited by their adaptor proteins to specific death signaling complexes where they are autocatalytically activated. Effectors caspases, including mammalian caspase-3 and the Drosophila caspase drICE, lack a long prodomain essential for the formation of activating complexes; thus, they require cleavage by activated initiator caspases to induce their catalytic activity (Fuentes-Prior and Salvesen, 2004; Kumar, 2007).

In vertebrates, caspases can be activated by either “extrinsic” or “intrinsic” pathways (Figure 1.3). Apoptotic signals through the extrinsic pathway initiate from a family of death receptors located on the cell surface; by contrast, the release of cytochrome c from mitochondria to the cytoplasm nucleates cell death signals via the intrinsic apoptosis pathway (Danial and Korsmeyer, 2004; Kumar, 2007; Rupinder et al., 2007).
1.1.2.1 The death receptor-dependent extrinsic pathway

Upon binding specific ligands, death receptors in the tumor necrosis factor superfamily, such as Fas (also known as CD95), TNFR-1, and DR3/4/5, undergo homooligomerization to form a caspase activating complex (Rupinder et al., 2007). In the case of Fas, the receptor undergoes trimerization and conformational changes following the binding of its ligand, FasL, facilitating the assembly of the Death-Inducing Signaling Complex (DISC) (Suda et al., 1993). In the DISC, the adaptor protein FADD uses its own Death Domain (DD) and Death Effector Domain (DED) to interact with the DD of Fas and the DED of initiator caspase, respectively (Figure 1.2B). The formation of this activating complex promotes initiator caspase-8 or -10 monomers to aggregate and activate by “induced proximity”, resulting in the subsequent stimulation of downstream effector caspases and the cell death of type I cells; however, Fas-induced initiator caspase activity is too weak to directly trigger death in type II cells. Instead, DISC-activated initiator caspases cleave the pro-apoptotic protein Bid to induce apoptosis with the aid of the intrinsic pathway (described in detail below). Hence, blockade of this signaling amplification loop by the anti-apoptotic protein Bcl-2 abrogates Fas-induced apoptosis in type II cells (Figure 1.3) (Danial and Korsmeyer, 2004; Scaffidi et al., 1998).

Signaling by the death receptor-dependent pathway plays an essential role in maintaining homeostasis of the immune system. Mutations in the gene encoding caspase-10 or Fas have been associated with autoimmunity, whereas caspase-8 deficiency causes defective lymphocytes and immunodeficiency (Danial and Korsmeyer, 2004).
1.1.2.2 The mitochondria-dependent intrinsic pathway

A wide range of cell-damaging stimuli, including UV radiation, oxidative stress, nutrient deprivation and chemotherapeutic agents, trigger the mitochondria-dependent pathway of cell death (Danial and Korsmeyer, 2004; Rupinder et al., 2007). In vertebrates, apoptotic signals regulate the expression of the Bcl-2 family proteins both transcriptionally and post-translationally (Borner, 2003; Tsujimoto, 2003). The balance between pro-apoptotic (e.g., Bax, Bak) and anti-apoptotic (e.g., Bcl-2, Bcl-XL) members of Bcl-2 family regulates the permeability of mitochondrial outer membrane (Chipuk and Green, 2008), and thereby controls the translocation of cytochrome c and other apoptotic regulators from the mitochondrial inter membrane space to the cytoplasm, the key initiation step of the intrinsic pathway (see below).

Once translocated, cytochrome c binds to the WD40 motifs of Apaf-1 and induces a conformational change, facilitating the assembly of a “wheel-like” protein complex consisting of seven Aapf-1 molecules (Figure 1.4) (Acehan et al., 2002; Yu et al., 2005; Yuan et al., 2011; Yuan et al., 2010). This heptamer then attracts caspase-9 through the CAspase Recruitment Domain (CARD) present in both Apaf-1 and caspase-9 to form an activating complex named the “apoptosome”, whereby individual caspase-9 molecules dimerize and activate by induced proximity (Danial and Korsmeyer, 2004). Active caspase-9 then cuts and activates its downstream effector caspases, caspase-3 and -7, which in turn mediate the proteolytic cleavage of various substrates and ultimately cell destruction (Danial and Korsmeyer, 2004; Inoue et al., 2009; Kumar, 2007). Other
mitochondrial apoptotic regulators, including Smac/DIABLO, Omi/Htra2, Apoptosis-Inducing Factor (AIF), and endonuclease G also assist to the cell dismantle during apoptosis (Danial and Korsmeyer, 2004). Once released to the cytoplasm, Smac/DIABLO and Omi/Htra2 sequester or cleave cellular Inhibitor of Apoptosis Proteins (IAPs) and thereby lift their inhibition on caspases (see section 1.2.2.1 on IAPs) (Chai et al., 2000; Du et al., 2000; Faccio et al., 2000; Verhagen et al., 2000), while AIF and endonuclease G translocate into the nucleus where they stimulate chromatin condensation and DNA fragmentation (Susin et al., 1999; van Loo et al., 2001).

Notably, in the intrinsic pathway, caspase-2 also functions as an initiator caspase in response to certain apoptotic stimuli, including heat shock, DNA damage, nutrient deprivation and ER stress (Krumschnabel et al., 2009). The exact mechanism of caspase-2 activation is still under debate, whereas it is generally recognized that caspase-2 engages in different “apoptosome-like” protein scaffolds for its activation in response to different stimuli (Krumschnabel et al., 2009). For example, the PIDDosome, which contains the p53-Induced protein with Death Domain (PIDD) and an adaptor protein, the RIP-Associated ICH-1/CED3 homologous protein with Death Domain (RAIDD), has been shown to mediate caspase-2 activation upon DNA damage (Read et al., 2002; Tinel and Tschopp, 2004). Once activated, caspase-2 cleaves the pro-apoptotic Bcl-2 family protein Bid to promote cytochrome c release and initiate the caspase cascade as stated above (Bonzon et al., 2006). Additionally, caspase-3 has been shown to cleave and activate caspase-2 in cytosolic extract, suggesting a possible positive feedback loop linking
caspase-2 and its downstream effector caspases (Swanton et al., 1999). However, caspase-2 dimerization, rather than cleavage, has been shown to be essential for its activation in vivo (Baliga et al., 2004). Therefore, the biological role of the caspase-3-mediated cleavage of caspase-2 is yet to be determined.

1.2 Apoptotic regulation targeted to caspases
Given the central role of caspases in apoptotic cell death, regulators that modulate either caspase activation or catalytic activity will greatly influence cell fate decisions (Callus and Vaux, 2007; Rudel, 1999). Natural caspase regulators are first identified from viruses; these proteins were found to inhibit caspases and cell death and thereby maximize viral replication in infected cells (Ray et al., 1992). Cellular homologues of viral caspase inhibitors were then subsequently identified based on peptide sequence homology, and have been shown to regulate apoptosis in physiological and pathological contexts (Callus and Vaux, 2007; Kumar, 2007; Rudel, 1999). Indeed, elevated levels of cellular caspase inhibitors have been observed in numerous human cancer cells, resulting in a refractory response to chemotherapeutics (Gyrd-Hansen and Meier, 2010; Safa et al., 2008). Meanwhile, post-translational modifications of caspases provide a different avenue to orchestrate apoptosis with other cellular processes, such as the cell cycle, metabolism, and DNA repair (Andersen et al., 2009; Cardone et al., 1998; Mannick, 2007; Martins et al., 1998; Nutt et al., 2005; Shin et al., 2005). In addition, proteins with domains capable of interacting with caspases may
directly interfere with the propagation of apoptotic signals and thus control cell death (Boldin et al., 1996; Cohen, 1997; Wang et al., 1994).

### 1.2.1 Viral caspase inhibitors

#### 1.2.1.1 CrmA

The cowpox Cytokine Response Modifier A (CrmA) was the first natural caspase inhibitor characterized (Ray et al., 1992). CrmA was initially shown to inhibit caspase-1, thus preventing the conversion of pro-interleukin 1 into its active form; the apoptosis-relevant substrate of CrmA was later found to be caspase-8 (Callus and Vaux, 2007; Zhou et al., 1997). This viral protein complexes with active caspases and uses its LVAD motif to serve as a suicide substrate, directly blocking the active site (Xue and Horvitz, 1995). Although CrmA originally appeared to block most vertebrate caspases, further examination suggests that it only inhibits caspase-1, -4, and -8 potently, and shows a very limited effect on other caspases at physiological concentrations (Callus and Vaux, 2007; Zhou et al., 1997). Because of its ability to inhibit caspase-8, CrmA has been shown to prevent cell death through the intrinsic pathway, including Fas- and TNFR-induced apoptosis (Miura et al., 1995; Tewari and Dixit, 1995).

#### 1.2.1.2 p35 and p49

The caspase inhibitors p35 and p49 originate from baculoviruses that were originally found to infect and block cell death in insect cells (Clem et al., 1991; Du et al.,
1999). Similar to CrmA, p35 and p49 also function as substrate inhibitors and can be cleaved by active caspases following the peptide sequence of DQMD and TVTD, respectively (Fisher et al., 1999; Zoog et al., 2002). The cleaved products then silence caspases by covalently linking themselves to caspases’ catalytic cysteine through a thioester bond (Xu et al., 2001). Notably, although both baculoviral proteins are broad spectrum caspase inhibitors capable of preventing cell death in nematodes, insects, and mammals, p49 appears to be more potent than p35. For some p35-insensitive caspases, including the mammalian initiator caspase-2 and the Drosophila initiator caspase Dronc, p49 effectively suppress their proteolytic activities (Callus and Vaux, 2007; Zoog et al., 2002).

1.2.1.3 v-FLIP

Viral FLICE-Inhibitory Protein (v-FLIP) can be distinguished from the other viral caspase inhibitors stated above by its inhibitory mechanism: it interferes with caspase activation rather than catalytic activity (Chang and Yang, 2000; Krueger et al., 2001). v-FLIP contains two DED motifs that are also present in FADD and the prodomain of caspase-8 (also known as FLICE) (Thome et al., 1997). As stated in section 1.1.2.1, by binding to the DED motifs of FADD, caspase-8 is recruited to the DISC where it activates by induced proximity. Mechanically, v-FLIP inhibits death receptor-mediated apoptosis by simply blocking FADD using its DED motifs, thus preventing the recruitment of caspase-8 to the DISC (Chang and Yang, 2000; Krueger et al., 2001). Loss-of-function analysis confirmed the anti-apoptotic ability of v-FLIP, as a mutant virus lacking of the
v-FLIP gene failed to confer apoptotic resistance to infected cells treated with the Fas ligand (Glykofrydes et al., 2000).

1.2.2 Cellular caspase inhibitors

1.2.2.1 Inhibitor of apoptosis proteins (IAPs)

Although Op-IAP and Cp-IAP were the first identified IAPs, the role of these two viral IAPs in caspase regulation is controversial (Birnbaum et al., 1994; Crook et al., 1993); their anti-death ability could only be observed in certain insect cells, and they appeared to inhibit caspase activation by modulating unknown targets located in the fly-specific signaling pathway upstream of the initiator caspase (Kaiser et al., 1998; LaCount et al., 2000; Seshagiri and Miller, 1997; Wright et al., 2005).

Nevertheless, cellular IAPs have been shown to directly control caspases in insects and vertebrates. So far, eight IAP members have been identified from the mammalian genome (Shi, 2002). XIAP, c-IAP1 and c-IAP2 have been shown to contain Baculovirual IAP Repeat (BIR) domains that interact with caspases, and a RING domain capable of mediating protein ubiquitination; however, subsequent studies have demonstrated that only XIAP can inhibit caspases at physiological concentrations (Shi, 2002). Consistent with this observation, in vitro assays also characterized XIAP as the most potent caspase inhibitor among all mammalian IAPs (Callus and Vaux, 2007).

Structural analyses further revealed how XIAP inhibit its targets, including the initiator caspase-9 and the effector caspase-3 and -7. XIAP bears three BIR domains and
a C-terminal RING domain. The BIR3 motif is essential for XIAP to block caspase-9, whereas the inhibition of effector caspases requires the linker region between BIR1 and BIR2 (Shi, 2002). Once processed, the newly exposed N-terminus of the caspase-9 small subunit allows XIAP to dock close to the active site of caspase-9 via the BIR3 motif (Srinivasula et al., 2001). This binding may then reduce the accessibility of caspase-9's active residue or prevent monomeric caspase-9 from further dimerization by steric hindrance (Callus and Vaux, 2007; Shi, 2002). In contrast, the linker region between BIR1 and BIR2 of XIAP directly occupies the active site of caspase-3 and -7, preventing the entry of substrates (Sun et al., 1999). Meanwhile, XIAP has also been suggested to control a cell’s apoptotic threshold by modulating the ubiquitination and stability of caspase-3 through its RING domain (Suzuki et al., 2001) (see section 1.2.3.4 below).

Notably, unlike their functional analogues in the fly, mammalian IAPs may only play an auxiliary role in apoptosis. *xiap* knockout mice show no apoptotic susceptibility nor developmental defect (Harlin et al., 2001), whereas reduced expression of DIAP1, the most potent fly IAP, causes autonomous apoptotic cell death in cultured cells and whole organisms (Details on DIAP1's function and regulation will be described later in section 1.3.1) (Igaki et al., 2002; Rodriguez et al., 2002). Nonetheless, many cancers have been shown to employ pathologically elevated levels of IAPs to evade apoptosis; IAPs, therefore, have emerged as novel therapeutic targets in cancer therapy (LaCasse et al., 2008).
1.2.2.2 c-FLIP

Similar to their viral counterpart, the inhibitory role of c-FLIP in death receptor-mediated apoptosis has been well characterized. Currently, three splice isoforms have been characterized in mammals (Bagnoli et al., 2010). c-FLIPs and c-FLIPs have only two DED domains, structurally mimicking v-FLIP. The longest isoform, c-FLIPL, bears a long C-terminal caspase-like domain in addition to its two DED motifs, while lacking any proteolytic activity (Bagnoli et al., 2010). Mechanically, c-FLIP uses its DED motifs to compete with caspase-8 for the DISC, thus blocking caspase-8 activation and death signaling by the extrinsic pathway (Bagnoli et al., 2010; Chang and Yang, 2000). Indeed, c-FLIP knockout renders cells more sensitive to Fas- and TNF-induced apoptosis, and the expression of c-FLIPL has been employed as an adverse prognostic factor in cancers (Safa et al., 2008; Yeh et al., 2000). Contradictorily, expression of c-FLIPL appears to activate rather than inhibit caspase-8 in some studies (Han et al., 1997; Shu et al., 1997). Although the precise cause for this discrepancy is still under investigation, it is generally believed that the caspase-8 to c-FLIPL ratio is crucial to determine the function of c-FLIPL in apoptotic regulation (Bagnoli et al., 2010).

1.2.3 Post-translational modification of caspase

1.2.3.1 S-nitrosylation

Nitric oxide (NO) donors inhibit TNFα-induced cell death in many cell types, including hepatocytes and colon cancer cells (Kim et al., 2002; Mannick, 2007; Wang et
al., 2010). As the active cysteine residue of many caspases have been shown to be susceptible to S-nitrosylation in vitro (Li et al., 1997), this raised the possibility that elevated NO may suppress cell death by modulating caspase activity via this post-translational modification. In support of this notion, several reports have demonstrated that caspases can be S-nitrosylated at their active site and thereby silenced in vivo, including caspase-1, -3, -8 and -9 (Choy and Pober, 2009; Dimmeler et al., 1997; Kim and Tannenbaum, 2004; Li et al., 1999; Rossig et al., 1999). Nitrosylation status in some cases seems to be modulated in response to apoptotic stimuli (Kim and Tannenbaum, 2004; Mannick et al., 1999). In healthy cells, S-nitrosylation of caspase-3 helps keep its caspase activity in check, and prevents its activation by promoting its association with acid sphingomyelinase. Upon the ligation of TNF ligands, caspase-3 is denitrosylated, permitting its activation and the proteolytic activity of its active site (Mannick et al., 1999).

Recently, S-nitrosylation has also been shown to regulate the caspase inhibitors XIAP and FLIP in vivo with opposite outcomes; S-nitrosylation compromises the inhibitory ability of XIAP, promoting neuronal loss in the progression of Parkinson's disease, but stabilizes FLIP by blocking its ubiquitination, preventing Fas-induced apoptosis (Chanvorachote et al., 2005; Tsang et al., 2009). As stated, S-nitrosylation appears to modify and modulate several key players in the apoptosis pathway. However, the universal impacts of S-nitrosylation on apoptosis have yet to be evaluated, mainly due to the technical constraints in the preservation and detection of this modification.
1.2.3.2 SUMOylation

Caspase-2, -7 and -8 are subject to the covalent attachment of Small Ubiquitin-related M0difier (SUMO) family proteins (Besnault-Mascard et al., 2005; Hayashi et al., 2006; Shirakura et al., 2005). Abrogation of caspase-2 SUMOylation in the prodomain appears to delay its activation, possibly by interfering with the interaction between caspase-2 and its activating protein (Shirakura et al., 2005). However, similar mutagenesis-based analyses have failed to detect any functional change in the activation or activity of caspase-7 and -8 (Besnault-Mascard et al., 2005; Hayashi et al., 2006). As SUMOylated caspases are exclusively detected in the nucleus, this type of post-translational modification might change the distribution of caspases, and therefore alter the apoptotic signaling circuit (Besnault-Mascard et al., 2005; Hayashi et al., 2006; Shirakura et al., 2005); however, the physiological relevance of this observation still needs to be determined.

1.2.3.3 Phosphorylation

Phosphorylation is the most common post-translational modification of caspases. As summarized in Table 1.1, phosphorylation has been shown to regulate many caspases, including mammalian caspase-1, -2, -3, -6, -7, -8, -9 and the Drosophila caspase Dronc; some of these caspases appear to be phosphorylated by different kinases at multiple sites, representing multiple caspase check points. Caspases functioning in either the intrinsic or extrinsic pathway are all susceptible to phosphorylation-mediated control, while most phosphorylation are targeted to the initiator caspases (e.g., caspase-
2, -8, -9 and Dronc), consistent with the idea that initiator caspases function as gatekeepers in apoptotic signaling pathways (see Table 1.1 for details). In general, phosphorylation suppresses caspase activity in vivo, either by impeding caspase activation (e.g. caspase-2) or by promoting caspase degradation (e.g. caspase-8) (Andersen et al., 2009; Peng et al., 2011). One exception is c-Abl-mediated phosphorylation of caspase-9 at Y153, which has been shown to be required for DNA damage-induced caspase-9 activation and consequent cell death (Raina et al., 2005).

Interestingly, the phosphorylation status of caspases appears to be modulated in response to cell cycle stage and nutrient status, thereby calibrating a cell’s sensitivity to apoptotic stimuli. The most prominent example is the CDK1-Cyclin B1-mediated suppression of apoptosis in mitosis. Surprisingly, initiator caspases of both the intrinsic pathway (e.g., caspase-2 and -9) and the extrinsic pathway (e.g., caspase-8) have all been shown to be phosphorylated and thus suppressed by CDK1-Cyclin B1 during mitosis (at S340, T125 and S38 in caspase-2, -9 and -8, respectively) (Allan and Clarke, 2007; Andersen et al., 2009; Matthess et al., 2010). Due to this multi-point inhibition of initiator caspases via mitotic specific phosphorylations, the cell’s apoptosis threshold rises, possibly for the prevention of unnecessary cell death that might otherwise result from normal mitotic processes, such as mitochondrial fission and cell detachment (Allan and Clarke, 2007; Andersen et al., 2009; Matthess et al., 2010).
Table 1.1: Phosphorylation-mediated caspase regulation

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Sites</th>
<th>Kinase/Phosphatase</th>
<th>Action</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>S376</td>
<td>PAK1</td>
<td>Activate</td>
<td>(Basak et al., 2005)</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>S157</td>
<td>CK2</td>
<td>Suppress</td>
<td>(Shin et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>S164</td>
<td>CaMKII/PP1</td>
<td>Suppress</td>
<td>(Nutt et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>S340</td>
<td>CDK1-CyclinB1/PP1</td>
<td>Suppress</td>
<td>(Andersen et al., 2009)</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>TBD</td>
<td>PKC</td>
<td>Activate</td>
<td>(Voss et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>S150</td>
<td>p38-MAPK/PP2A</td>
<td>Suppress</td>
<td>((Alvarado-Kristensson et al., 2004), (Alvarado-Kristensson and Anderson, 2005))</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>S257</td>
<td>ARK5</td>
<td>Suppress</td>
<td>(Suzuki et al., 2004)</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>S30, T173, S279</td>
<td>PAK2</td>
<td>Suppress</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>S38</td>
<td>CDK1-CyclinB1</td>
<td>Suppress</td>
<td>(Matheus et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>T263</td>
<td>RSK2</td>
<td>Suppress¹</td>
<td>(Feng et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Y310</td>
<td>TBD</td>
<td>Activate²</td>
<td>(Jia et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>S364</td>
<td>p38-MAPK</td>
<td>Suppress</td>
<td>(Alvarado-Kristensson et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Y397</td>
<td>Src, Lyn/SHP1</td>
<td>Suppress</td>
<td>(Cursi et al., 2006), (Jia et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Y465</td>
<td>Lyn/SHP1</td>
<td>Suppress</td>
<td>(Jia et al., 2008)</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>TBD⁵</td>
<td>PKA</td>
<td>Suppress</td>
<td>(Martin et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>T125</td>
<td>ERK1/2, DYRK1A,</td>
<td>Suppress</td>
<td>(Allan et al., 2003), (Allan and Clarke, 2007), (Seifert and Clarke, 2009), (Laguna et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDK1-CyclinB1,</td>
<td></td>
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<td></td>
<td></td>
<td>p38-MAPK/PP1</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>S144</td>
<td>PKC</td>
<td>Suppress</td>
<td>(Brady et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>S153</td>
<td>c-Abl</td>
<td>Activate</td>
<td>(Raina et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>S196⁴</td>
<td>Akt</td>
<td>Suppress</td>
<td>(Cardone et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>S348⁵</td>
<td>CK2</td>
<td>Suppress</td>
<td>(McDonnell et al., 2008)</td>
</tr>
<tr>
<td>Dronc</td>
<td>S130</td>
<td>CaMKII</td>
<td>Suppress</td>
<td>(Yang et al., 2010)</td>
</tr>
</tbody>
</table>

TBD, to be determined

¹ by promoting caspase degradation via the ubiquitination-proteasome system
² by recruiting SHP1 to dephosphorylate inhibitory sites, Y397 and Y465
³ S99, S183 and S195 were initially indentified but nonphosphorylatable mutants did not support their necessity. The relevant site is therefore still to be determined.
⁴ human specific; not conserved in other species
⁵ murine specific; not conserved in other species

Another paradigm for the phosphorylation-mediated regulation of caspases is demonstrated by the inhibitory phosphorylation of caspase-2 at S164 (Nutt et al., 2005).

This CaMKII-mediated phosphorylation appears to be regulated by cellular metabolism; caspase-2 S164 is phosphorylated in cells with sufficient nutrients to prevent its
activation, while it is dephosphorylated and therefore activated upon energy crisis (Nutt et al., 2005). In Chapter 2 of this dissertation, we further discovered that a conserved metabolic signaling pathway also controls the activation of Drosophila initiator caspase Dronc via an inhibitory phosphorylation.

Of note, phosphorylation status is usually controlled by the balance between the addition and the removal of a phosphate group; therefore, characterization of a caspase’s corresponding kinases and phosphatases and their regulations will lead to new insights into the apoptotic regulatory network.

### 1.2.3.4 Ubiquitination

Ubiquitin, a small regulatory protein containing 76 amino acids, has been shown to be covalently linked to the lysine residues of substrates by a series of enzymatic reactions, altering the substrate’s stability, protein-protein interaction, subcellular localization and enzymatic activity. In the ubiquitin conjugation process, substrate specificity is controlled by an E3 ubiquitin ligase, which possesses either a RING or HECT domain that is required for the last step of ubiquitin conjugation (Hochstrasser, 1996).

Among all of the identified vertebrate caspases, caspase-3, -7, -8 and -10 have ever been suggested to be modified by ubiquitin (Huang et al., 2000; Jin et al., 2009; McDonald and El-Deiry, 2004; Suzuki et al., 2001; Tan et al., 2006); however, the ubiquitination of caspase-7 and -10 has not been observed in vivo. Caspase-3 ubiquitination has been shown to be catalyzed by two different ubiquitin ligases, XIAP and the SCFβTrCP complex (Suzuki et al., 2001; Tan et al., 2006). Overexpression of either
ligase stimulates the ubiquitination and degradation of caspase-3, causing resistance to both etoposide- and TNF-induced apoptosis (Suzuki et al., 2001; Tan et al., 2006). Similarly, fly apoptosis has been shown to be tightly regulated by protein ubiquitination as well (Bergmann, 2010; Hay and Guo, 2006; Kornbluth and White, 2005; Kumar, 2007). DIAP1, the most dominant IAP in *Drosophila*, suppresses both the initiator caspase Dronc and the effector caspase drICE through ubiquitination-mediated protein degradation (Igaki et al., 2002; Wilson et al., 2002) (see section 1.3.1.1 for details).

In contrast, a recent report demonstrated an alternative mode of ubiquitination-mediated caspase regulation (Jin et al., 2009). In this study, caspase-8 appeared to be ubiquitinated by a cullin3 (CUL3)-based E3 ligase in response to the ligation of TRAIL, a type of death ligand, to its DR4 receptor. This modification did not alter the assembly of the known DISC components, including DR4, FADD and caspase-8. Rather, ubiquitinated caspase-8 was recruited by p62, an ubiquitin binding protein, and activated in a novel DISC complex containing p62. Strikingly, abrogation of the assembly of this novel DISC complex by either p62 or CUL3 RNAi significantly dampened caspase-8 activity upon TRAIL treatment, demonstrating the necessity of this complex (and the ubiquitination of caspase-8) in TRAIL-induced apoptosis (Jin et al., 2009). This finding has extended the current view of the extrinsic apoptotic pathway, and was the first demonstration of a stimulating role for ubiquitination in caspase regulation. Further investigations using other death ligands, such as Fas and TNF, will be required to examine the generality of this finding.
1.2.4 Other caspase regulators

It is worth mentioning that most caspases have multiple splice isoforms, including caspase-1, -2, -4, -5, -6, -7, -8, -9, and -10. Some caspase isoforms are missing their catalytic domain, but still contain motifs responsible for their recruitment to the corresponding activating complex (Boldin et al., 1996; Wang et al., 1994). This type of caspase deletion mutant is capable of occupying the docking sites in the activating complex and can, therefore, prevents the activation of its wild-type counterpart, functioning as a caspase inhibitor in a way similar to the FLIP. This notion is supported by the identification of inhibitory isoforms of caspase-2 and caspase-8, which lack a catalytic domain and demonstrate anti-apoptotic effects when ectopically expressed (Boldin et al., 1996; Wang et al., 1994).

The complexity of the apoptotic regulatory network keeps growing as novel caspase regulators are identified. For example, NOL3 (also known as ARC), a novel apoptosis repressor with a CARD domain, has been shown to block both intrinsic and extrinsic apoptosis and associates with caspase-2 and -8 in vivo (Koseki et al., 1998; Nam et al., 2004). CARP, a newly characterized protein with a RING motif, was found to block cell death induced by death ligands and physically interact with caspase-8 and -10 (McDonald and El-Deiry, 2004). Their molecular mechanisms have yet to be determined, but the interaction of NOL3 and CARP with caspases might in part account for their inhibitory effects.
Collectively, the fact that cells express multiple caspase regulators demonstrates that apoptosis is a highly regulated process. Elucidation of the apoptotic regulatory network, especially how it is derailed in diseases, will benefit the identification of novel therapeutic targets.

1.3 *Drosophila as a model animal to study apoptosis*

With its genetic tractability and easily-observed phenotypes, *Drosophila* has been used widely to study programmed cell death *in vivo* (Richardson and Kumar, 2002). A recently established genome-wide RNAi screens in fly cells further enables scientist to characterize genes of unknown function, making *Drosophila* a distinct model system that can bridge research between biochemistry and genetics. Since the fly apoptotic signaling pathway shares considerable similarity, with less redundancy, to its vertebrate counterpart, *Drosophila* provides an ideal system to elucidate the physiological functions of major apoptotic players and dissect the apoptotic network (Richardson and Kumar, 2002). Indeed, several novel apoptotic regulators recently discovered from genome-wide RNAi screens carried in *Drosophila* cell, have been shown to modulate mammalian apoptosis as well (Chew et al., 2009; Yi et al., 2007). Again, these findings fully demonstrate that *Drosophila* can be a fruitful system for the study of the apoptotic regulatory network.
1.3.1 The *Drosophila* apoptotic signaling pathway

As shown in Figure 1.5, the core apoptotic machinery is well conserved throughout evolution, whereas the regulatory phase of apoptosis differs among species. The core apoptotic machinery consists of caspases and their corresponding activating proteins, which initiate the execution phase of apoptosis by forming the apoptosome, leading to the activation of apical caspases and the eventual dismantling of the cell. Given the destructive nature of the core apoptotic machinery, it is tightly controlled by the balance of multiple apoptotic agonists and antagonists during the regulatory phase to prevent unnecessary death, while ensuring timely cell destruction when needed.

The nematode has a simple linear pathway that shows strikingly similarity to the vertebrate intrinsic pathway (Kumar, 2007). In both cases, a pro-apoptotic Bcl-2 protein containing a single BH3 domain is transcriptionally upregulated upon receipt of a death stimulus, lifting the suppression mediated by anti-apoptotic Bcl-2 family proteins and allowing the execution phase of apoptosis to initiate (Figure 1.5). Therefore, the balance between pro- and anti-apoptotic Bcl-2 family proteins (and the subsequent release of cytochrome c in vertebrates) appears to govern the key regulatory step in both nematodes and vertebrates.

In contrast, although the involvement of mitochondria and Bcl-2 family proteins in *Drosophila* apoptosis is still under a great deal of controversy, mainly due to conflicting results generated from different experimental conditions (see section 1.3.2.1), IAPs have been generally recognized as the major regulator for both developmental and
stress-induced apoptosis in *Drosophila* (Kornbluth and White, 2005). Here, the prevailing consensus view of the *Drosophila* apoptotic signaling pathway in most tissues is described.

1.3.1.1 The executor: *Drosophila* caspases and their activating proteins

Thus far, seven caspases have been identified from the fly genome (Hay and Guo, 2006; Kumar, 2007). The domain structures of representative caspases are depicted in Figure 1.2. Dronc (Dorstyn et al., 1999a), Dredd (Chen et al., 1998) and Strica (Doumanis et al., 2001) are believed to function as initiator caspase due to their long N-terminal prodomains, implicating that they can oligomerize and thus self-activate in the apoptosome. The remaining four caspases, drICE (Fraser and Evan, 1997), DCP-1 (Song et al., 1997), DAMM (Harvey et al., 2001) and DECAY (Dorstyn et al., 1999b), are effector caspases lacking a long prodomain and require an initiator caspase for their activation.

Among all of the characterized caspases, Dronc and its downstream effector caspases, drICE and DCP-1, have been clearly linked to apoptosis (Dorstyn et al., 1999a; Fraser and Evan, 1997; Kaiser et al., 1998; Song et al., 1997). In contrast, due to the lack of specific mutants, the physiological roles of Strica and DAMM are currently unknown, while genetic analyses demonstrate that mutations in Dredd and DECAY have no (or only very limited) impact on cell death, indicating they may be primarily involved in other biological processes rather than apoptosis (Kumar, 2007). Genetic ablation of Dronc and drICE causes pupal lethality and resistance to developmental- and stress-induced apoptosis in most tissues, whereas DCP-1 knockout only delays cell death in
certain tissues (e.g. larval eye disc) without inducing any developmental abnormality (Kumar, 2007).

Dronc and drICE, therefore, appear to be the primary apoptotic caspases, while DCP-1 shares functional redundancy with drICE; together, these three caspases mediate most, but not all, apoptotic cell death in Drosophila (Kumar, 2007). Interestingly, biochemical assays have revealed the special substrate specificity of Dronc. Unlike other caspases, which only cleave substrates following an Asp residue, Dronc can autoprocess after a Glu residue as well (Hawkins et al., 2000). Meanwhile, Dronc is only sensitive to the baculoviral caspase inhibitor p49 (but not p35) which is capable of blocking all Drosophila effector caspases including drICE and DCP-1 (Zoog et al., 2002).

Similar to the mammalian initiator caspases, the activation of the Drosophila initiator occurs in an “apoptosome-like” protein complex consisting of Dronc and Dark (Drosophila Apaf-1 Related Killer) (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). The Drosophila apoptosome, unlike its mammalian counterpart, contains eight (instead of seven) adaptor proteins, and, strikingly, neither its formation nor function appears to require cytochrome c, despite the fact that Dark bears several WD40 motifs potentially capable of binding cytochrome c (Dorstyn and Kumar, 2008; Yu et al., 2006; Yuan et al., 2011). Thus, the fly apical caspase Dronc constitutively undergoes Dark-dependent processing in S2 cells (Muro et al., 2002), which potentially stabilizes Dronc as a homodimer and stimulates its proteolytic activity (Dorstyn and Kumar, 2008; Shi, 2002; Snipas et al., 2008). However, this autonomous caspase activity is largely
neutralized by Drosophila IAPs in healthy cells (see section 1.3.1.2 below), allowing caspases to mediate their non-apoptotic roles while not initiating cell destruction (Huh et al., 2004a; Igaki et al., 2002; Meier et al., 2000; Muro et al., 2002; Rodriguez et al., 2002).

1.3.1.2 The gatekeeper: Drosophila IAPs

As previously stated, normal fly cells express DIAP1, the most potent member in the Drosophila IAP family, to prevent unnecessary cell death (Igaki et al., 2002; Muro et al., 2002; Rodriguez et al., 2002). Although the molecular mechanisms of DIAP1-mediated caspase suppression are still under active investigation, DIAP1’s central role in controlling Drosophila apoptosis is well established in both cultured cells and transgenic animals (Hay and Guo, 2006; Kornbluth and White, 2005; Kumar, 2007). In a variety of experimental systems, including S2 cells, reducing DIAP1 levels is enough to trigger apoptosis (even in the absence of a death stimulus), demonstrating DIAP1’s functions as a gatekeeper to silence autonomous death signaling in healthy cells (Igaki et al., 2002; Meier et al., 2000; Muro et al., 2002; Rodriguez et al., 2002; Wang et al., 1999b; Wilson et al., 2002).

Mechanically, DIAP1’s RING domain, which mediates substrate ubiquitination, has been proven to be required for cell death suppression; however, several different, and somewhat contradictory, models have been suggested to explain how DIAP1 inhibits different caspases. Generally, DIAP1 reduces cellular caspase activity by blocking the caspase’s catalytic site and by functioning as an E3 ligase to promote caspase ubiquitination and degradation. Alternatively, in the case of drICE,
ubiquitination blocks the entry of substrates through steric hindrance or changing its subcellular localization. (Bergmann, 2010; Ditzel et al., 2008; Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002).

In addition to DIAP1, three other fly proteins harbor a BIR domain, including DIAP2 (Hay et al., 1995), dBRUCE (Vernooy et al., 2002) and DETERIN (Jones et al., 2000), suggesting that they may also function as caspase inhibitors; indeed, initial reports have demonstrated their anti-apoptotic activities against certain stimuli in cultured cells, whereas genetic removal did not impair apoptosis, reflecting their minor or redundant (if any) role in controlling developmental-induced apoptosis in the examined tissues. Recently, dBRUCE has been shown to be subjected to autophagic degradation, thereby releasing the proteolytic activity of DCP-1 during Drosophila oogenesis (Nezis et al., 2010). This observation reveals that Drosophila IAPs can modulate caspases to fulfill their non-apoptotic roles.

1.3.1.3 The sensor: the RHG family proteins

To ignite the Drosophila caspase cascade, cells lift the DIAP1-mediated caspase suppression through the RHG (Reaper, Hid and Grim) family proteins, a group of death activators that are capable of binding and neutralizing IAPs through their IAP Binding Motif (IBM) (Goyal et al., 2000). To date, six fly proteins have been shown to harbor an IBM, including Reaper, Hid, Grim, Sickle, Jafranc2 and dOMI (Bergmann, 2010). Among them, Reaper, Hid and Grim were the first identified and most studied members.
Upon receipt of an apoptotic signal, fly cells transcriptionally and post-translationally upregulate the activity of the RHG family proteins (Bergmann et al., 2002; Kurada and White, 1998; Nordstrom et al., 1996). Since RHG proteins and caspases share the same docking sites on DIAP1, the upregulation of RHGs prevents DIAP1 from binding caspases (Yoo et al., 2002). The RHG family proteins also decrease DIAP1 levels by stimulating its autoubiquitination and by global translational inhibition (Kornbluth and White, 2005; Yoo et al., 2002). Without DIAP1’s inhibition, activated Dronc mediates the proteolytic cleavage of downstream effector caspases drICE and DCP-1, leading to apoptotic cell death (Hawkins et al., 2000; Xu et al., 2006).

Similarly, upon the activation of the intrinsic apoptosis pathway, mammalian cells release two mitochondrial proteins, Smac/DIABLO and Omi/Htra2, into the cytoplasm. These proteins have been proved to bind IAPs through their IBM and suppress IAPs, lowering the apoptotic threshold in mammalian cells (Chai et al., 2000; Du et al., 2000; Faccio et al., 2000; Verhagen et al., 2000).

### 1.3.2 Other emerging cell death regulators in apoptosis and beyond

Many additional cell death regulators have been identified from genetic screen carried out in transgenic lines or cultured cells. Unlike the well studied apoptotic regulators stated above, these novel factors were initially found to modulate cell death in a context-dependent manner. Interestingly, the activation of RHG proteins and caspases were traditionally considered an indicator of apoptotic cell death, but this notion is now being challenged by accumulating evidence of “apoptotic” regulators.
playing roles in “non-apoptotic” types of Drosophila cell death, such as autophagy.

Therefore, these observations raise the following question: When a particular mode of cell death demands both “apoptotic” (e.g., caspases and RHG proteins) and “non-apoptotic” (e.g., accumulation of atg-bound autophagosomes) factors, is it considered as “apoptosis”? Can the factors that modulate this type of death be termed “apoptotic” regulators? Given the fact that flies express much fewer proteins than mammals, using a handful of proteins (e.g., caspases) to deal with numerous types of cell death appears to be an “economically” inevitable choice.

Despite their fuzzy functional niches and unclear physiological roles, the initial discovery of Drosophila cell death regulators helped elucidate how fly cells make the most important decision to live or die. Here, some novel death regulators, together with others that have been long speculated to function in Drosophila cell death, are described.

1.3.2.1 Mitochondria: not just a bag of cytochrome c

Although the mitochondrion has been shown to be an apoptotic regulatory platform in vertebrates, integrating both pro- and anti-apoptotic signals relayed by the Bcl-2 family proteins to control cytochrome c release, the contribution of mitochondria to Drosophila apoptosis remains elusive.

Cytochrome c (and therefore its source, mitochondria) was initially suggested to be involved in the formation of Drosophila apoptosome, as the fly caspase-activating protein Dark possess multiple WD40 domains that have been shown to physically interact with cytochrome c in vitro (Rodriguez et al., 1999; Yu et al., 2006). In support of
its role in caspase regulation, cytochrome c has been suggested to be required for caspase-dependent spermatogenesis and retina formation during fly development (Arama et al., 2006; Mendes et al., 2006). However, subsequent reports show that drICE activity during spermatogenesis appears to be Dronc- and Dark-independent, while the deletion line utilized to study eye formation lacks cytochrome c and two other flanking genes as well, raising the possibility that decreased caspase activity might be secondary to impaired mitochondrial functions (Huh et al., 2004b). Indeed, rigorous structural evidence and several in vivo mutagenesis studies all demonstrated that neither caspase activation nor cell death required the presence of cytochrome c, dimming the role of mitochondria in controlling the fate of Drosophila cells (Dorstyn and Kumar, 2008; Yu et al., 2006; Yuan et al., 2011).

Nevertheless, a growing body of evidence still supports the involvement of mitochondria in Drosophila cell death. Interestingly, several apoptotic regulators, including RHG proteins, DIAP1, Dronc and drICE, have long been known to localize on or near to mitochondria for unknown purposes (Claveria et al., 2002; Dorstyn et al., 2002; Freel et al., 2008; Haining et al., 1999; Olson et al., 2003; Thomenius et al., 2011). To answer this, recent studies suggest that RHG protein-mediated mitochondrial remodeling is required to fully activate caspases during apoptosis (Abdelwahid et al., 2007; Goyal et al., 2007; Thomenius et al., 2011). Overexpression of Reaper and Hid causes mitochondrial fragmentation, possibly by activating mitochondrial fission proteins (e.g., Drp-1) or by inhibiting fusion proteins (e.g., dMFN). Strikingly, inhibiting
mitochondrial remodeling dampens Reaper’s caspase-inducing activities, suggesting the necessity of mitochondrial fragmentation in *Drosophila* apoptosis (Abdelwahid et al., 2007; Goyal et al., 2007; Thomenius et al., 2011). However, although one report suggests that changes in mitochondria morphology occur prior to caspase activation (Goyal et al., 2007), another contradictorily shows that pan-caspase inhibitor zVAD blocks such remodeling (Abdelwahid et al., 2007), questioning the causative relationship between these two phenomena. Nevertheless, no matter which scenario is true, these observations suggest a role for mitochondria in *Drosophila* cell death.

As cytochrome c is unlikely involved, how do mitochondria pave the road for fly cell death? The recent identification of *Drosophila* Omi (dOMI) might provide a clue for this unsettled issue (Khan et al., 2008). Upon apoptotic signals, changes in mitochondrial ultrastructures may activate or release dOMI and other unknown factors from mitochondria, permitting them to mediate their pro-apoptotic functions. Alternatively, compromised mitochondria function resulting from the perturbation of remodeling may cause an energy imbalance and induce cell death. In support of this, abrogation of mitochondrial fusion has been shown to induce mitochondria dysfunction (Chen et al., 2005), which potentially activates *Drosophila* p53-dependent cell cycle arrest and cell death (Mandal et al., 2005).

### 1.3.2.2 *Drosophila* Bcl-2 family proteins

With a domain structure similar to mammalian Bcl-2 proteins, the *Drosophila* proteins Debcl/Drob-1/dBorg-1/dBok and Buffy/dBorg-2 have been speculated to control
apoptosis in the fly. Generally, when overexpressed, Debcl and Buffy show pro- and anti-apoptotic effects respectively. Recent updates further reveal that Debcl primarily resides on mitochondria, whereas Buffy localizes to the endoplasmic reticula (Doumanis et al., 2007). However, specific deletion lines generated recently indicate that they control developmental apoptosis in a very limited number of tissues, such as ovary, and instead may be involved in radiation-induced apoptosis (Sevrioukov et al., 2007; Tanner et al., 2011). Interestingly, knockout of either Debcl or Buffy causes abnormal mitochondria morphology in ovary (Tanner et al., 2011). Future studies on how Drosophila Bcl-2 proteins regulate the mitochondrial fission/fusion will be of interest.

1.3.2.3 Metabolic regulators

Alterations in cellular metabolism, such as glycolysis and the TCA cycle, have been shown to affect cell viability through many key apoptotic players in vertebrate cells (Hammerman et al., 2004). Recently, a number of metabolic regulators have surfaced in several independent screens for novel Drosophila apoptotic regulators, supporting the notion that cellular metabolism directly impinges on apoptosis (Chew et al., 2009; Wang et al., 2008; Yi et al., 2007). Hits identified from these screens function in a variety of critical metabolic pathways, including ATP generation, fatty acid biosynthesis, polysaccharide metabolism, and the TCA cycle, suggesting the presence of multiple checkpoints coordinating cell death with metabolic status.

Interestingly, succinyl CoA synthase (CG1065) and citrate synthase (CG14740), two critical TCA cycle enzymes identified from two independent screens, are shown to
exert opposite effects on cell death (Chew et al., 2009; Yi et al., 2007). Reduction in succinyl CoA synthase sensitizes *Drosophila* S2R+ cells to DIAP1 antagonist-induced apoptosis (Chew et al., 2009); in contrast, knockdown of citrate synthase alleviates cell death induced by DIAP1 dsRNA in Kc cells (Yi et al., 2007). The molecular mechanisms underlying these two observations are not investigated in the original reports; however, given their opposite effects, it is unlikely that their regulations can be attributed to the globally compromised activity of the TCA cycle. Nevertheless, the fact that they modulate apoptosis induced by the loss of DIAP1 suggests they act downstream of DIAP1, most likely by directly modulating caspases or Dark.

Another TCA cycle enzyme, malate dehydrogenase (CG7998), was identified in a genetic screen for loss-of-function mutations that block developmental apoptosis in the salivary gland (Wang et al., 2008). In their discussion, the authors suggested that the disruption of mitochondrial function might account for the apoptotic resistance in embryos lacking malate dehydrogenase; however, no related data was shown (Wang et al., 2008). Although how these aforementioned metabolic regulators control *Drosophila* apoptosis remains unclear, our study presented in Chapter 2 provides a direct link between NADPH metabolism and caspase activation, demonstrating the first characterized DIAP1-independent regulation of *Drosophila* apoptosis through the inhibitory phosphorylation of Dronc.
1.3.2.4 UPS modulators

As described in section 1.3.1, ubiquitination appears to be the molecular basis of many apoptotic regulatory events in Drosophila. Almost all of the key apoptotic regulators, including RHG family proteins, IAPs and caspses, are subjected to ubiquitination-based regulations. DIAP1’s RING domain, which mediates protein ubiquitination, has been shown to be indispensible for its regulatory ability (Wilson et al., 2002). It’s therefore not surprising to know that modulators of the Ubiquitin-Proteasome System (UPS) affect apoptotic cell death in Drosophila.

In addition to metabolic regulators, the screen mentioned previously also identified Tango7 and its mammalian analogue PCID1 as novel apoptotic regulators (Chew et al., 2009). Ablation of Tango7 or PCID1 expression caused resistance to UV-induced apoptosis, which can be at least partly attributed to reduced expression levels of initiator caspase (Dronc and caspase-9 in the fly and human cells respectively). Because of their PCI domain, which usually presents in the regulatory subunits of proteasome, Tango7 and PCID1 were suggested to modulate Dronc and caspase-9 protein levels through compromised proteasome activity. (Chew et al., 2009). Subsequent studies on molecular mechanisms will help verify this assumption.

Recently, a deubiquitinase, SCNY/USP36 (CG5505), was found to play an essential role in fly eye and ovary development (Ribaya et al., 2009). Loss-of-function mutants of SCNY exhibited enhanced cell death associated with a decrease in DIAP1 levels, suggesting an anti-apoptotic activity for SCNY. Consistent with the previous
result, overexpression of a long isoform of SCNY inhibited death induced by Reaper and Grim, but not Hid and a DIAP1 hypomorph. The original report neither pinpointed the direct target of SCNY nor biochemically characterized how SCNY modulated the ubiquitination chain on its target. However, another concurrent report confirmed SCNY’s deubiquitinase activity by showing that SCNY removed ubiquitin from histone H2B \textit{in vivo} and \textit{in vitro} to modulate chromatin structure and thereby prevent stem cells from premature differentiation (Buszczak et al., 2009). Since SCNY is also expressed in differentiated cells, these two studies collectively suggest a certain amount of versatility for SCNY’s role in different cell phases.

In sum, with well established genetic approaches and newly developed biochemical assays, the \textit{Drosophila} model provides a unique platform, allowing researchers to promptly characterize molecular mechanisms of novel observations and then examine the physiological relevance at the whole organism level. \textit{Drosophila} apoptosis exhibits both conserved and distinct features. As numerous \textit{Drosophila} apoptotic regulators have been proven to be functionally conserved in mammals, with some of them even involve in the oncogenesis of human cancers, a better understanding of the fly apoptotic network potentially benefits both basic and clinical research.

\section*{1.4 Metabolic regulation of apoptosis in vertebrate cells}

Apoptosis plays an essential role in maintaining tissue homeostasis, which is balanced by the generation of new functional cells and the elimination of unneeded ones. Interestingly, cellular metabolic rate appears to correlate well with cell fate. For
instance, in the case of hematopoietic cells, growth factors (e.g., Interleukin-3) stimulate cell proliferation as well as glucose uptake; conversely, following growth factor withdrawal, cells reduce their metabolic rate and eventually die in apoptosis. This perfect co-fluctuation raises an intriguing hypothesis that cellular metabolism may modulate the cell’s decision to proliferate or die.

Indeed, in the past decade, accumulating evidence has demonstrated that the regulatory circuit of metabolism and apoptosis are actually intertwined. Particularly, several enzymes initially found to function in major metabolic pathways, including glycolysis, TCA cycle, and the pentose phosphate pathways (PPP), have been shown to suppress key apoptotic regulators, collectively preventing cell death in a nutrient-rich environment. As cells are constantly challenged by numerous intrinsic and extrinsic stresses that damage cellular components, this metabolism-mediated survival signal may indicate the presence of enough resources for repair, warranting cells a “second chance” before they commit suicide. Conversely, apoptosis will be initiated to eradicate damaged cells when their repair systems are compromised by metabolic perturbation. Here we summarize some confirmed links to exemplify the crosstalk between cellular metabolism and apoptosis.

### 1.4.1 The PI3K/Akt/GSK3β pathway – a signaling hub that controls both metabolism and apoptosis

The PI3K/Akt/GSK3β pathway has been shown to be a major survival signaling circuit, regulating both glucose metabolism and apoptosis (Plas and Thompson, 2005).
Upon stimulation of growth factors, active Akt itself directly enhances glucose uptake by facilitating the translocation of the glucose transporter to the cell surface (Hill et al., 1999; Rathmell et al., 2003; Wang et al., 1999a; Wieman et al., 2007; Wofford et al., 2008; Zhou et al., 2008), and reduces the activity of the pro-apoptotic Bcl-2 family protein Bad through inhibitory phosphorylation (Datta et al., 1997; del Peso et al., 1997).

Furthermore, stimulated Akt also phosphorylates and inactivates GSK3β to mediate several corresponding metabolic and apoptotic adjustments: (A) Removal of GSK3β-mediated phosphorylation of the voltage-dependent anion channel (VDAC), permitting unphosphorylated VDAC to interact and recruit hexokinases to the mitochondrial outer membrane. Mitochondria bound hexokinases then phosphorylate glucose to glucose-6-phosphate (G6P) to facilitate its utilization (Pastorino et al., 2005), and prevent Bax binding and the subsequent cytochrome c release (Pastorino et al., 2002). (B) Abrogation of GSK3β-mediated phosphorylation of Mcl-1, an anti-apoptotic Bcl-2 protein, prevents its degradation through the UPS (Maurer et al., 2006). Mcl-1 is then able to neutralize other pro-apoptotic Bcl-2 proteins, including Noxa and Bim, resulting resistance to apoptosis (Alves et al., 2006; Coloff et al., 2011a). (C) Inhibition of GSK3β-mediated phosphorylation of TIP60, an acetyltransferase, decreases its enzymatic activity. TIP60 has just recently been shown to mediate p53 acetylation and thereby facilitate p53-regulated induction of the pro-apoptotic Bcl-2 protein Puma. Therefore, this axis of PI3K/Akt/GSK3β signaling can block Puma expression by blocking its transcription (Charvet et al., 2011).
1.4.2 Glucose metabolism and its suppression of apoptotic regulators

Interestingly, the anti-apoptotic functions of the Akt pathway appear to rely on, at least in part, an elevated glucose metabolism. Overexpression of glucose transporter, Glut-1, has been shown to prevent cell death induced by hypoxia and growth factor deficiency (Lin et al., 2000; Rathmell et al., 2003). Meanwhile, hexokinases, the first enzyme in the glycolysis pathway, can inhibit apoptosis by either blocking Bax translocation or by binding to VDAC to prevent cytochrome c release (Azoulay-Zohar et al., 2004; Pastorino et al., 2002). The presence of glucose and glycolysis flux also has been proven to be required to maintain leukemic cell viability after growth factor withdrawal by stimulating Mcl-1 synthesis and by suppressing the activation of Bax and the expression of Puma (Coloff et al., 2011a; Coloff et al., 2011b; Rathmell et al., 2003).

In addition to gating cytochrome c release through the modulation of Bcl-2 proteins, metabolic flux can also control apoptosis at a post-mitochondrial stage. NADPH generated from the PPP keeps cytochrome c in its reduced state, interfering with its binding to Apaf-1 and the assembly of the apoptosome, which is required for the intrinsic apoptosis pathway (Vaughn and Deshmukh, 2008).

1.4.3 NADPH-mediated regulation of oocyte cell death

Another paradigm for the metabolic regulation of apoptosis has been uncovered in our laboratory utilizing the Xenopus oocyte extract system. As depicted in Figure 1.6, glucose uptake stimulates the production of NADPH via the PPP, resulting in CaMKII-mediated phosphorylation of Xenopus caspase-2 at S135. This phosphorylation interferes
with the interaction between caspase-2 and its activating protein RAIDD, and thus prevents the assembly of the caspase-2 activating complex. Upon nutrient deprivation, caspase-2 S135 phosphorylation is decreased, lifting the blockade of caspase-2 activation. Activated caspase-2 will then cleave Bid and thereby stimulate cytochrome c release from mitochondria, resulting in oocyte apoptosis through the intrinsic pathway (Nutt et al., 2005).

1.5 Rationale and specific aims

Thus far, no DIAP1-independent apoptotic regulation has ever been characterized in Drosophila. Recently, by genetic or whole-genome RNAi screens, several groups have independently demonstrated that many metabolic regulators can still modulate caspase activation upon the loss of DIAP1 (Chew et al., 2009; Wang et al., 2008; Yi et al., 2007), implicating the existence of a signaling circuit coordinating Drosophila metabolic status and caspase activation. However, the direct link has yet to be determined.

As described in section 1.4, in most cases, metabolism regulates apoptosis in most cases by modulating the Bcl-2 family proteins and cytochrome c release. Nevertheless, Drosophila Bcl-2 family proteins and cytochrome c are suggested not to be involved in fly apoptosis; therefore, these mitochondria-related links between metabolism and apoptosis are unlikely to control Drosophila cell death.

Intriguingly, the prodomain of the Drosophila initiator caspase Dronc shares peptide sequence homology with that of caspase-2 (Dorstyn et al., 1999a). As we previously demonstrated that NADPH metabolism controls caspase-2 through an inhibitory phosphorylation on its prodomain (Nutt et al., 2005), it raises an interesting
possibility that NADPH may also control fly apoptosis by modulating Dronc phosphorylation.

The studies presented in this dissertation represent a first attempt to address whether NADPH-mediated metabolic control of apoptosis is conserved in vertebrates and invertebrates, in an effort to uncover a hitherto unknown mechanism for regulating *Drosophila* apoptosis. Ultimately, with the advantages provided by this model system, we aim to elucidate factors/pathways that are universally involved in the metabolic regulation of apoptosis.
Figure 1.1: Morphological and biochemical features of apoptotic cell death.

Figure 1.1: (A-B) Phase-contrast microscopy capturing the formation of apoptotic bodies in SKOV-4 human ovarian cancer cells and *Drosophila* S2 cells undergoing apoptosis. Arrows: membrane blebbing and disrupted cells. (C-D) Transmission electron microscopic analysis of normal (C) and apoptotic (D) HEK293T cells. Arrows: condensed and marginalized chromatin. (E) Genomic DNA extracted from normal (Lane 2) and apoptotic SKOV-4 cells (Lane 3). DNA was cleaved by nucleases into fragments of ~200 base pairs during apoptosis, resulting in a “ladder” pattern. Adapted from (Edelweiss et al., 2008) (A & E) and (Lei et al., 2009) (C & D) with permission from PLoS One as an Open-Access Journal.
Figure 1.2: Protein domain structures of representative caspases and adaptor proteins.

Figure 1.2: (A) Primary caspases functioning in *Drosophila* and vertebrate apoptosis. All caspases bear a catalytic domain that will be autoprocessed into a large (p20, ~20 kDa) and a small (p10, ~10 kDa) fragments upon activation. Initiator caspases also harbor a long prodomain that contains a CAspase Recruitment Domain (CARD) or Death Effector Domain (DED) capable of mediating protein-protein interaction. (B) Adaptor proteins that bind to initiator caspases to form the activating protein complexes. In the formation of the activating protein complexes for caspase-8 and -2, a Death Domain (DD) is required for FADD and RAIDD to interact with death receptors and PIDD, respectively. Although Dark’s protein domain structure exhibits a striking similarity to Apaf-1, and bears WD40 domains capable of binding cytochrome c, the *Drosophila* apoptosome does not contain cytochrome c. NOD, nucleotide binding and oligomerization domain; NBD, part of NOD, nucleotide binding domain (see text for details). Figure shown in A is adapted from (Kumar, 2007) with permission from the Nature Publishing Group.
Figure 1.3: The extrinsic and intrinsic apoptotic pathways.

Figure 1.3: Caspase-2 and caspase-9 are activated in their activating protein complexes, the PIDDosome and the apoptosome, respectively, to mediate cell death via the mitochondria-dependent intrinsic pathway. Pro- and anti-apoptotic members of the Bcl-2 family proteins regulate the release of cytochrome c from mitochondria, the key regulatory step in the intrinsic pathway, while caspase-2 activates the pro-apoptotic Bcl-2 protein Bid through proteolytic cleavage to initiate this process. Upon the ligation of a death ligand on the cell surface, caspase-8 is activated in the DISC, consisting of trimerized death receptors, adaptor protein FADD, and caspase-8, to trigger a death signal via the death receptor-dependent extrinsic pathway. In type I cells, activated caspase-8 cleaves and directly activates caspase-3, which is utilized by both the intrinsic and extrinsic pathways to mediate the dismantling of the cell through the proteolytic cleavage of several cellular substrates. Note in type II cells, caspase-8-mediated Bid cleavage is required for the crosstalk between the intrinsic and extrinsic pathway (see text for details). From (Kurokawa and Kornbluth, 2009), with permission from Elsevier.
Figure 1.4: Vertebrate and Drosophila apoptosome.

Figure 1.4: (A) Linear diagrams of Dark (upper) and Apaf-1 (lower) are shown with seven color-coded functional domains and linkers in gray; the same color code is applied to B and C. (B) Structures of the apoptosome platform formed by Dark (upper) and Apaf-1 (lower) are shown in a top view with the CARD domains omitted for clarity. The Drosophila apoptosome is formed by eight Dark subunits in the absence of cytochrome c, while the vertebrate apoptosome has seven cytochrome c-bound Apaf-1 molecules. (C) Putative conformational changes in Apaf-1 upon cytochrome c binding, protruding its CARD domain from the apoptosome platform to from a disk-like complex with caspase-9’s CARD. (D) Top and side views of the 3D map of the apoptosome-caspase-9 CARD complex. The apoptosome platform is shown in blue, and a disk-like structure formed by the CARD domains of Apaf-1 and caspase-9 is shown in magenta. Adapted from (Yuan et al., 2011) (A, Dark structure and B) and (Yuan et al., 2010) (A, Apaf-1 structure, C and D), with permission from Elsevier.
Figure 1.5: Drosophila and vertebrate apoptotic signaling pathways.

Figure 1.5: Signaling pathways during the execution phase of apoptosis are evolutionarily conserved; however, compared to the fly, vertebrate systems employ additional control mechanisms during the regulatory phase. The caspase cascade induced by the initiator caspases and their activating complex is responsible for the execution phase of apoptosis in both vertebrates and invertebrates. The key regulator of Drosophila apoptosis is DIAP1, the predominant fly IAP with a RING domain, which silences caspases in healthy cells and is neutralized by the RHG family proteins upon receipt of an apoptotic stimulus. Downregulation of DIAP1 triggers caspase activation and apoptosis even in the absence of a death signal. In contrast, genetic ablation of the DIAP1 analogue, XIAP, shows no obvious abnormality. Instead, the major control point of the vertebrate intrinsic apoptotic pathway is mediated by members of the Bcl-2 family of proteins. Bcl-2 proteins regulate the release of cytochrome c from mitochondria, which subsequently initiates the execution phase of the vertebrate intrinsic apoptosis pathway; however the roles of cytochrome c and Bcl-2 proteins in Drosophila apoptosis remain elusive (see text for details).
**Figure 1.6: NADPH-mediated metabolic control of caspase-2**

**Figure 1.6:** Caspase-2 activation in *Xenopus* oocyte extract is controlled by an inhibitory phosphorylation at S135. NADPH produced from the pentose phosphate pathway stimulates this CaMKII-mediated phosphorylation and thus silences caspase-2. Upon nutrient deprivation, this phosphorylation is decreased by a mechanism yet to be confirmed, permitting caspase-2 to oligomerize and activate in its activating complex, the PIDDosome, by binding to an adaptor protein, RAIDD. PP1 can dephosphorylate caspase-2 S135 *in vitro*, but neither its binding with caspase-2 nor its catalytic activity appears to be regulated by NADPH metabolism. Once activated, caspase-2 cleaves Bid. Truncated Bid then translocates to the mitochondria and stimulates cytochrome c release, fating cells to die by the intrinsic apoptosis pathway.
Chapter 2  Metabolic regulation of Drosophila apoptosis through inhibitory phosphorylation of Dronc

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2.1 Introduction

Accumulating evidence suggests that cellular metabolism impinges directly upon the decision to initiate cell death (Nutt et al., 2005; Rathmell et al., 2003; Yi et al., 2007; Yuneva et al., 2007; Zhao et al., 2008). In vertebrate cells, glucose metabolism and apoptosis are mutually regulated, at least in part, through the Bcl-2 family proteins which control mitochondrial cytochrome c release, a key process in vertebrate intrinsic apoptosis (Kluck et al., 1997; Liu et al., 1996; Rathmell et al., 2003; Zhao et al., 2008). An additional paradigm for metabolic regulation of apoptosis is exemplified by caspase 2, which can be activated upon NADPH deprivation in Xenopus oocytes and is suppressed through phosphorylation in nutrient replete oocytes (Nutt et al., 2005). When activated, caspase-2 cleaves and activates the Bcl-2 family member Bid, to promote cytochrome c release from mitochondria and subsequent cell death.

Drosophila apoptosis is, instead, regulated by the balance between the inhibitor of apoptosis proteins (IAPs) and a group of pro-apoptotic regulators known as the RHG (Reaper, Hid and Grim) proteins (Kornbluth and White, 2005). The Drosophila initiator caspase Dronc is believed to be constitutively activated through autoprocessing by its activating protein, the Drosophila Apaf-1 homolog Dark (Igaki et al., 2002; Muro et al., 2002; Rodriguez et al., 2002). However, this continuous apoptotic signaling is largely
antagonized in healthy cells by DIAP1, which suppresses the catalytic activity of Dronc and meditates its degradation through ubiquitination to prevent unnecessary cell death (Meier et al., 2000; Muro et al., 2002; Wilson et al., 2002; Yoo et al., 2002). The RHG proteins, transcriptionally-upregulated following receipt of apoptotic stimuli, compete with DIAP1 for its binding site on caspases and decrease DIAP1 levels by stimulating its autoubiquitination, allowing the apoptotic signaling to propagate throughout the caspase cascade and initiate cell death (Goyal et al., 2000; Kornbluth and White, 2005; Wang et al., 1999b; Yoo et al., 2002).

Recent RNAi-based screens have revealed that several metabolic regulators are involved in control of caspase activation (Yi et al., 2007), suggesting that fly apoptosis may be subject to metabolic control. Although mitochondrial release of cytochrome c does not appear to be required for caspase-dependent cell death in most Drosophila cells tested (Abdelwahid et al., 2007; Dorstyn and Kumar, 2008; Dorstyn et al., 2002), the regulation of vertebrate caspase 2 by NADPH levels raised the interesting possibility that Drosophila caspases might also be directly controlled by NADPH metabolism.

We show here that the Drosophila initiator caspase Dronc is inhibited by phosphorylation at S130 in response to abundant NADPH and that abrogation of this phosphorylation by a point mutation renders this caspase refractory to metabolic control. These observations identify cellular NADPH levels as a novel gatekeeper that sets the threshold for Drosophila apoptosis through modulating Dronc activation, and
suggest that such regulatory mechanisms are evolutionarily-conserved and operate in somatic cells as well as in germ cells.

2.2 Materials and methods

2.2.1 dsRNA synthesis

Table 2.1: Sequences of oligonucleotide primers used to generate dsRNA templates.

<table>
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<th>Gene</th>
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<th>Forward primer (5’ to 3’)*</th>
<th>Reverse primers (5’ to 3’)*</th>
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<td>Dronc</td>
<td>CG8091</td>
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<td>Dark</td>
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<td>GGCACGGACAAAAATGATTTGATTTAGTG</td>
<td>CCGAGATTTGGAATCCCGGAGGAGCCAT</td>
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<td>drICE</td>
<td>CG7788</td>
<td>ATGGAGCCGACTACAAATGAGAGAACC</td>
<td>ACGAATGCTCTCAGAATATGCTATTAGT</td>
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<td>DIAP1</td>
<td>CG12284</td>
<td>ATGGCATCTTTTCTAGTCTATTC</td>
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<td>Men</td>
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<td>#1F: GGCCTTAACTACTAACAGGAAAC</td>
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<tr>
<td></td>
<td></td>
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*T7 promoter sequence (gaattaatcagctactatagggaga) was added to the 5’ end of each primer.

Drosophila cDNA clones were purchased from Drosophila Genomics Resource Center at Indiana University Bloomington Campus. Double-stranded RNAs were synthesized according to the protocol from the Drosophila RNAi Screening Center at Harvard medical school with modifications. Briefly, for each reaction 1-2 μg of DNA template with T7 polymerase binding site was mixed with other reagents from the MEGAscript T7 kit (Ambion #1334) in an RNAase-free tube to a final volume of 30 μl. Reaction was performed at 37°C for 6-18 h, followed by LiCl precipitation according to
the manual. Purified dsRNA dissolved in RNAase free TE (pH 8.0) was heated to 70°C for 15 min and then incubated at room temperature for 30 min. The quality and quantity of synthesized dsRNA was examined by both spectrophotometry (OD.260/280 ratio of 2) and agarose gel electrophoresis. Annealed dsRNA aliquots were stored at -80°C. Primers used to generate RNAi templates can be found in Table 2.1.

2.2.2 Cell culture

*Drosophila* Schneider’s S2 cells were maintained in standard Schneider’s medium [Schneider’s medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen)]. For RNAi in S2 cells, cells were treated with three successive two-day treatments of 80 nM dsRNA in standard Schneider’s medium unless otherwise specified in the figure legends. Subpassage was performed every two days. Medium were supplemented with 50 μM zVAD as needed. Samples for immunoblotting were lysed in 2× SDS sample buffer, followed by sonication. To measure caspase activity, treated cells were lysed in hypotonic buffer (50 mM HEPES, pH 7.7, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 10 μM cytochalasin B, 1× Roche complete protease inhibitor, 10 mM Na₃P₂O₇). Lysates were incubated with 90 μl DEVDase buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol) and the substrate Ac-DEVD-pNA (200 μM, Enzo) at 37°C. Absorbance was measured at 405 nm and normalized to the protein amount inputted. Cellular NADPH levels were measured by NADP/NADPH quantification kit (BioVision) according to the manual.
2.2.3 Transient and stable transfections

S2 cells were transfected by Effectene (QIAGEN) according to the manual. For transient expression, cells were transfected with 0.4-0.8 μg of DNA. For stable transfections, cells were cotransfected with 0.05 μg of GFP expression plasmids and 0.5 μg of plasmids containing a hygromycin B resistant gene and the copper-inducible gene of interest. Transfected cells were selected in standard Schneider’s medium supplemented with 125 μg/ml hygromycin B for more than 3 weeks.

2.2.4 Cell viability assay

Apoptosis was triggered with 40 μM CHX in S2 cells cultured in standard Schneider’s medium, 40 nM DIAP1 dsRNA in serum free Schneider’s medium or indicated concentrations of DHEA in glucose-free medium [HBSS supplemented with 1× essential and non-essential amino acid mixture (Invitrogen), pH 7.0 by NaOH] in the presence or absence of 5 mM dimethyl L-malate (Sigma) or 25 μM orlistat (Sigma). Cell viability was analyzed by flow cytometry for PI-positive cells in PBS with 0.5 μg/ml PI or pictures taken under microscopes. To test malate’s protection in S2 stable lines, cells were induced for 8 h with 150 μM CuSO₄ in standard Schneider’s medium in the presence or absence of 5 mM malate or 50 μM zVAD, followed by the addition of 40 μM CHX for another 8 h. Flow cytometry was used to score GFP-positive cells. Means ± SD from three independent experiments are shown.
2.2.5 Metabolic labeling of phosphoproteins in S2 cells

S2 cells expressing FLAG-Dronc prodomain were pretreated with 100 μM DHEA ± 5 mM malate for 2 h in glucose-free medium supplemented with 50 μM zVAD. After pretreatment, cells in each well were labeled with 1 mCi $^{32}$P-orthophosphate (PerkinElmer) for 4 h in the same medium. Treated cells were then lysed in lysis buffer (40 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 4 mM EDTA, 1 mM DTT, 10 mM Na$_4$P$_2$O$_7$, 10 mM NaF, 2 mM Na$_3$VO$_4$, and 1× Roche complete protease inhibitor). Dronc prodomain were immunoprecipitated with FLAG beads (Sigma), and analyzed by immunoblotting and autoradiography.

2.2.6 Co-immunoprecipitation

Co-immunoprecipitation assays were performed as previously described (Quinn et al., 2000; Yang et al., 2005). In brief, lysates from transfected cells were incubated with FLAG beads (Sigma) at 4°C for 2 h. Precipitates and whole cell lysates (WCL) were then analyzed by immunoblotting with indicated antibodies.

2.2.7 In vitro kinase assay

Bacterially-expressed GST fusion Dronc prodomain (WT, S127A and S130A) bound to glutathione-Sepharose beads was incubated in kinase buffer (10 mM Tris-HCl at pH 7.2, 0.1 mM ATP, 2 μCi $^{32}$P-ATP, 10 mM MgCl$_2$ and 1 mM DTT at pH 7.2) with CaMKII (CalBiochem) for 1 h at room temperature followed by SDS-PAGE, coomassie blue stain and autoradiography.
2.2.8 Antibodies

Rabbit anti-DIAP1 serum (1:500) and Dronc pS130 phospho-specific antibody (1:1,000) were raised against synthetic peptides, and the latter was then purified as previously described (Nutt et al., 2009). Anti-Dronc (1:1,000) antibody was raised in rabbit against purified recombinant Dronc large domain (p20). Anti-FLAG (Sigma, 1:3,000) and anti-actin (Santa Cruz, 1:2,000) antibodies were used. Purified anti-DIAP1 (1:1,000), anti-drICE (1:3,000), and anti-bursiconα (1:1,000) antibodies were generous gifts from Masayuki Miura (Univ. of Tokyo), Paul Friesen (Univ. of Wisconsin-Madison) and Benjamin H. White (NIH) respectively.

2.2.9 Purification of recombinant Dronc

pDEST15 plasmids (Invitrogen) encoding N-terminally GST-tagged Dronc were transformed into Escherichia coli strain BL21-AI (Invitrogen). Overnight cultures were grown at 37°C and then diluted 1:10 in LB broth. Expression of protein was induced at 30°C with L-arabinose (2g/L) for 1 h when the O.D. (600 nm) of the diluted cultures reached 0.7. After harvesting, bacteria were sonicated in lysis buffer (50 mM HEPES, pH 7.7, 100 mM NaCl, 1× Roche complete protease inhibitors). Recombinant proteins were then purified from the lysates using Glutathione Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions.
2.2.10 Transgenic flies and phenotyping

Transgenic lines from stock centers are listed in Table 2.2. UAS-Dronc transgenic flies were generated according to standard protocols. The integration sites in different lines were mapped to individual chromosomes. Expression of FLAG-tagged Dronc in different lines was driven by indicated Gal4 drivers. UAS-p35 flies were obtained from John York (Duke University). Progeny from the indicated crosses was categorized into unexpanded (UEX), partially expanded (PEX) and totally expanded (EX) based on criteria previously described (Luan et al., 2006). Individual lines and all crosses were maintained at 18°C, unless otherwise specified. To score wing expansion defects, newly-eclosed progeny were transferred into clean vials within 3 h after eclosion and then incubated at 18°C overnight. To examine the darkening process in the fly abdomen, flies were transferred within 1 h after eclosion and then incubated at 25°C for 3 h. Hemolymph was extracted and analyzed as previously described (Luan et al., 2006).

<table>
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<th>Synonyms</th>
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2.2.11 Statistical test

Data from flow cytometry-based experiments were analyzed by unpaired two-tailed Student’s t test. Nominal data were analyzed by Chi-square analysis.
2.3 Results

2.3.1 Study of metabolic influence on Drosophila apoptosis in cultured cells

2.3.1.1 Inhibition of NADPH production via the pentose phosphate pathway triggers apoptosis in Drosophila S2 cells

To elucidate a potential regulatory role for cellular NADPH levels in controlling Drosophila apoptosis, we treated Drosophila Schneider’s S2 (S2) cells with varying concentrations of dehydroepiandrosterone (DHEA), an allosteric inhibitor of glucose-6-phosphate dehydrogenase (G6PDH), to inhibit NADPH production via the pentose phosphate pathway (PPP). DHEA treatment induced dosage-dependent cell death as evidenced by a decrease in cell density and a increase in the percentage of propidium iodide (PI)-positive cells, both of which were significantly suppressed by the addition of dimethyl L-malate (hereafter referred to as malate), a cell permeable malate analog that elevates NADPH levels in conjunction with malic enzyme (Men) (Figure 2.1A and B). These results suggest that NADPH could modulate cell death in Drosophila cells. Notably, malate also blocked DHEA-induced membrane blebbing, a typical characteristic of apoptotic cell death, as cells treated with malate maintained a healthy morphology, even when exposed to 100 μM DHEA for 24 h (Figure 2.1A, right panel).

To confirm that DHEA-induced cell death was occurring by apoptosis, we examined DEVDase (effector caspase-like) activity in lysates from DHEA-treated cells. As shown in Figure 2.1C, DHEA treatment stimulated DEVDase activity in S2 cells, while this
increase was largely suppressed by malate, indicating that malate alleviates DHEA-induced caspase activation and consequent apoptosis.

To further elucidate how DHEA and malate regulate apoptosis, NADPH levels in lysates from treated cells were measured. Strikingly, DHEA treatment decreased cellular NADPH levels by 60%, while the addition of malate significantly restored this reduction (Figure 2.1D), consistent with the idea that these two reagents control apoptosis through modulating cellular NADPH levels. Indeed, abrogation of malate-induced NADPH production by downregulation of Men demolished the protective effects of malate (See section 2.3.1.5).

2.3.1.2 Drosophila initiator caspase Dronc mediates apoptosis induced by NADPH deprivation

Among seven identified caspases in Drosophila melanogaster, Dronc, Dredd, and Strica (Dream) are categorized as initiator caspases due to their long prodomains (Hay and Guo, 2006). Moreover, Dronc, with the aid of Dark, has been shown to mediate most developmental and stress-induced apoptosis in Drosophila, including spontaneous caspase activation induced by the loss of DIAP1 (Chew et al., 2004; Daish et al., 2004; Kanuka et al., 1999; Rodriguez et al., 2002; Rodriguez et al., 1999; Waldhuber et al., 2005; Xu et al., 2005; Zhou et al., 1999). Therefore, we first examined whether NADPH deprivation induces apoptosis through Dronc and Dark.

Upon DHEA treatment, S2 cells pretreated with control (GFP) dsRNA underwent apoptotic cell death, while this cell death was completely abrogated in cells
pretreated with either Dronc or Dark dsRNA, as shown by a constant cell density over 24 h of DHEA treatment in Dronc or Dark-deficient cells (Figure 2.2A). Immunoblotting also confirmed that both Dronc and Dark dsRNA molecules were able to downregulate their targets (Figure 2.2B). Consistent with previous reports, silencing of Dronc or Dark also blocked apoptosis induced by DIAP1 dsRNA or cycloheximide (CHX), indicating that DHEA, as well as these two apoptosis-inducing reagents, use the same signal transduction pathway to mediate cell death (Figure 2.2A). The processing of caspases in DHEA-treated cells was also analyzed by immunoblotting. As shown in Figure 2.2C, DHEA treatment resulted in the cleavage of Dronc and its downstream effector caspase, drICE, in cells treated with control (GFP) dsRNA, while the activation of drICE was totally blocked in Dronc-deficient cells, supporting the necessity of Dronc for DHEA-induced apoptosis. Interestingly, DHEA-induced cleavage of Dronc and drICE was greatly suppressed by the addition of malate (Figure 2.2D), suggesting that metabolic control targets either Dronc or an upstream regulator of Dronc. Taken together, these data strongly suggest that metabolic deficiencies induce apoptosis via the canonical cell death pathway mediated by Dark, Dronc and its downstream effector caspase, drICE.

2.3.1.3 Malate protects S2 cells from apoptosis upon the loss of DIAP1

Results presented above demonstrated that malate blocked DHEA-induced apoptosis. As DIAP1 dsRNA, CHX and DHEA all appeared to promote death through Dark and Dronc, we hypothesized that malate might also suppress apoptosis induced by DIAP1 dsRNA and CHX. Surprisingly, malate significantly protected S2 cells from
apoptosis resulted from the loss of DIAP1. As shown in Figure 2.3A and B, malate alleviated the decrease in cell density and the upsurge in caspase activity induced by DIAP1 dsRNA. Interestingly, immunoblotting using DIAP1 antibody revealed that malate neither interfered with DIAP1 degradation nor increased DIAP1 protein levels (Figure 2.3 F and G), suggesting that a novel metabolically-regulated cell death checkpoint lies downstream of DIAP1.

In addition, we examined malate’s effects on CHX-induced apoptosis. Due to the short in vivo half-life of DIAP1 (~30 min), blockade of de novo protein synthesis with CHX causes a decrease in endogenous DIAP1 pools, resulting in the accumulation of processed Dronc and subsequent cell death (Igaki et al., 2002; Muro et al., 2002; Rodriguez et al., 2002; Wilson et al., 2002). Similarly to DIAP1 dsRNA, CHX-induced apoptosis was also significantly suppressed by the addition of malate, as evidenced by malate-mediated restoration of cell density and suppression of caspase activity (Figure 2.3C and D).

2.3.1.4 Dronc is a direct target subject to metabolic control

We also monitored the cleavage of different caspases along the Dronc signaling pathway. Notably, the addition of malate significantly delayed the activation of Dronc in CHX-treated cells. Most Dronc was processed after 6 h of CHX treatment, resulting in the cleavage of its downstream substrate, drICE (Figure 2.3E, left panel). In contrast, Dronc-mediated cleavage of drICE was completely blocked in cells co-treated with malate (Figure 2.3E, right panel), indicating that malate markedly suppresses CHX-
induced increases in the proteolytic activity of Dronc. However, malate appeared to have no effect on the signaling pathways upstream of Dronc. The addition of malate affected neither the steady-state levels nor the half-life of DIAP1 (Figure 2.3F and G), indicating that metabolic control is not likely to act through the regulation of DIAP1. As shown in Figure 2.3H, CHX globally inhibits translation, including synthesis of the RHG proteins. Therefore, the RHG proteins are very unlikely to be involved in malate’s, as they are largely controlled through transcriptional induction.

Taken together, these data strongly suggest that Dronc, rather than the upstream RHG proteins and DIAP1, is directly suppressed by metabolism. Since factors involved in apoptotic control downstream of DIAP1 are largely unknown, we wished to decipher the signaling pathway underlying this novel regulation. Therefore, we utilized CHX as an apoptotic stimulus, bypassing control of RHG proteins and DIAP1 and focusing on downstream metabolic control of Dronc.

2.3.1.5 Malate’s protection against apoptosis requires NADPH generation by malic enzyme

Intracellular malate can either enter the tricarboxylic acid (TCA) cycle in mitochondria or be metabolized by malic enzyme in the cytoplasm to produce NADPH. To confirm that malate’s ability to protect cells from CHX-induced apoptosis required NADPH production, we knocked-down Men using Men dsRNA. Interestingly, downregulation of Men sensitized S2 cells to CHX, as extensive membrane blebbing and decreased cell density were observed in Men-deficient cells after 6 h of CHX treatment,
whereas most cells pretreated with control (GFP) dsRNA remained healthy at this time point (Figure 2.4A and B, 6 h). These data suggest that Men helps to set the threshold for apoptosis. More importantly, malate’s anti-apoptotic ability was largely abrogated by the silencing of Men, indicating apoptosis is not inhibited by malate *per se* but by downstream metabolic effects dependent upon Men (Figure 2.4A and B, 12 h). Similar results were obtained with dsRNA molecules directed against a different sequence of Men (data not shown). The efficacy of Men dsRNA was also assessed by immunoblotting (Figure 2.4C).

Malate’s effects were also assessed by monitoring of caspase activity. As expected from the results above, the addition of malate suppressed CHX-induced caspase activation in cells pretreated with control dsRNA, but had very limited effects in Men-deficient cells (Figure 2.4D). Malate is metabolized by malic enzyme to produce NADPH and pyruvate (Frenkel, 1975); the latter enters the TCA cycle (Jeffrey et al., 1996). In Men-deficient cells, malate would still have been expected to enter the TCA cycle, yet its protective effects were largely abolished. Collectively, these data supported the notion that *Drosophila* apoptosis can be controlled by altering cellular NADPH levels.

### 2.3.1.6 Cellular NADPH levels modulate death-inducing activity of Dronc through an inhibitory phosphorylation at Dronc S130

Since our data suggested that Dronc could be directly modulated by cellular NADPH levels, we wished to elucidate the molecular mechanism underlying this metabolic control. Interestingly, when the peptide sequence of Dronc was compared
with those of different vertebrate caspases, Dronc was found to be most similar to caspase-2 within its prodomain (Cooper et al., 2009). As metabolism regulates the activation of caspase-2 through phosphorylation in its prodomain, we first used metabolic phosphate labeling to determine how cellular NADPH levels might affect the phosphorylation of Dronc prodomain. As shown in Figure 2.5A, inhibition of the PPP by DHEA was accompanied by dephosphorylation of ectopically-expressed Dronc prodomain, while the addition of malate overrode this decrease. DHEA-induced dephosphorylation of Dronc was not inhibited by the pan-caspase inhibitor, zVAD, suggesting that Dronc dephosphorylation is a caspase-independent event occurring prior to caspase activation. Experiments using a series of Dronc mutants further revealed that mutation at S130, but not the nearby S127, suppressed phosphorylation of the Dronc prodomain to a sub-detectable level. These data suggest that malate functions to maintain phosphorylation of the Dronc prodomain at S130.

To monitor the phosphorylation status of endogenous Dronc in response to cellular NADPH levels, we generated an affinity-purified phospho-specific antibody (Dronc pS130) directed against this phosphosite. To validate the antibody, FLAG-tagged full-length Dronc carrying a mutation (C318A) to prevent autoprocessing was immunoprecipitated and treated with λ-phosphatase. As shown in Figure 2.5B, Dronc pS130 antibody only recognized phosphorylated Dronc, in that λ-phosphatase treatment abolished recognition by this antibody. More importantly, the phospho-antibody was unable to recognize Dronc (S130A/C318A) that had been mutated at S130 to render it
non-phosphorylatable, indicating that this antibody specifically detects Dronc phosphorylated at S130 (Figure 2.5C). Notably, malate treatment reversed the DHEA-induced decrease in signal detected by the phospho-specific (Dronc pS130) antibody, revealing that phosphorylation of endogenous Dronc at S130 is indeed controlled by NADPH levels in S2 cells (Figure 2.5D).

Given that an increase in NADPH levels resulted in phosphorylation of Dronc at S130, we wished to characterize the physiological significance of this phosphorylation. Towards this end, sub-lethal levels of full-length wild-type (WT) Dronc or Dronc S130A phosphomutant were induced in stably transected S2 cells constitutively expressing GFP. We then examined the responsiveness of these cells to malate upon CHX treatment by monitoring the loss of GFP by flow cytometry. Similar assays have been utilized in other systems to assess cell viability (Zimmermann et al., 2002). To validate this assay in our system, CHX-treated cells collected at various time points were used to assess the percentage of GFP-positive cells by flow cytometry and then lysed to measure cellular DEVDase activity by caspase assay (Figure 2.5E). We found that the percentage of GFP-positive cells decreased as caspase activity increased. Moreover, blocking CHX-induced caspase activity with zVAD inhibited the accompanying loss of GFP signal, indicating that the loss of GFP was due to caspase-dependent cell death. Thus, this measure was used as a proxy to assess cell viability in the following experiments.

Notably, cells transfected with either empty vector or WT Dronc expression plasmids remained sensitive to malate, as cell viability was partially restored by malate
treatment (Figure 2.5F, white and gray bar). By contrast, malate was unable to protect cells expressing Dronc S130A from CHX-induced apoptosis. These data indicate a vital role for phosphorylation of Dronc at S130 in metabolic control of apoptosis (Figure 2.5F, black bar). Note that WT Dronc and Dronc S130A proteins were expressed at comparable levels as assessed by immunoblotting (Figure 2.5G).

2.3.1.7 CaMKII phosphorylates Dronc at S130 and is essential for NADPH-mediated survival

We next attempted to identify the kinase responsible for phosphorylation at Dronc S130 in vivo. PKA, CK1 and calcium/calmodulin-dependent kinase (CaMK), were predicted to phosphorylate Dronc at S130 based on the known consensus sequences of these kinases and the peptide sequences flanking this site. PKA and CK1, however, appeared not to be involved in this metabolic control of Dronc, as neither chemical inhibitors (H-89 for PKA and IC261 for CK1) at concentrations known to inhibit the Drosophila kinases (Mennella et al., 2009; Zhao et al., 2007) nor dsRNA targeted against these two kinases, blocked malate’s ability to protect from CHX-induced apoptosis (Figure 2.6A and B).

Interestingly, downregulation of CaMKII, but not CaMKI, by dsRNA was able to abolish malate’s protective effect; cells pretreated with CaMKII dsRNA underwent apoptosis upon CHX treatment even in the presence of malate, whereas this apoptotic cell death was greatly suppressed by malate in cells pretreated with GFP or CaMKI dsRNA (Figure 2.6B). Cell lysates from CaMKII-deficient cells were also analyzed for
phosphorylation at S130 by immunoblotting. As shown in Figure 2.6C, malate was unable to restore phosphorylation of Dronc at S130 in cells pretreated with CaMKII dsRNA. Furthermore, *in vitro* kinase assays also revealed that CaMKII could directly phosphorylate the Dronc prodomain at S130, as a mutation at S130 abrogated CaMKII-mediated phosphorylation of the recombinant GST-tagged Dronc prodomain, while both recombinant WT and S127A Dronc prodomain, but not GST, were susceptible to CaMKII (Figure 2.6D). Collectively, these data strongly suggest that CaMKII is the relevant kinase that phosphorylates Dronc S130 in intact cells.

Interestingly, we have reported previously that CaMKII is responsible for caspase 2 phosphorylation in response to changes in NADPH levels in *Xenopus* oocytes. Thus, our data presented here also suggest that a similar regulatory circuit linking metabolism and apoptosis is evolutionarily conserved in vertebrates and *Drosophila*.

### 2.3.1.8 NADPH-mediated phosphorylation of Dronc interferes its activation by Dark rather than its catalytic activity

We reasoned that this metabolically-regulated phosphorylation might either directly suppress Dronc's catalytic activity, or interfere with its activation. We first tested the effect of S130 phosphorylation on Dronc activation. The interactions between Dronc and its activating protein, Dark, or its inhibitory protein, DIAP1, were examined by co-immunoprecipitation. Both catalytically-inactive Dronc (C318A) and phosphomutant (C318A/S130A) were specifically precipitated in association with the Dark CARD domain (Dark1-411) in transfected S2 cells. Notably, Dronc phosphomutant
(C318A/S130A) displayed a stronger interaction with Dark, suggesting that phosphorylation of the Dronc prodomain could hamper the formation of the *Drosophila* apoptosome by decreasing the interaction between Dronc and Dark (Figure 2.7A). In contrast, we observed no change in the interaction between Dronc and DIAP1, regardless of the phosphorylation status of Dronc S130 (Figure 2.7B).

Since recombinant CaMKII could phosphorylate Dronc at S130 *in vitro*, this allowed us to determine if phosphorylation of Dronc could alter its catalytic activity. Recombinant Dronc was purified from bacteria where high concentrations of Dronc could trigger its Dark-independent dimerization and autoprocessing, activating its proteolytic activity as previously described (Dorstyn and Kumar, 2008; Muro et al., 2004). Immunoblotting indicated the presence of two processed Dronc species in our preparation: Pr1 resulted from a cleavage between the small and large subunit, which allows Dronc to form a stable homodimer with greatly enhanced catalytic activity, and the prodomain released upon full processing of Dronc (Figure 2.7C, middle panel) (Dorstyn and Kumar, 2008; Muro et al., 2002; Muro et al., 2004; Yan et al., 2006). 

35S-labeled full-length drICE was then used as a substrate for Dronc that had been either mock-treated or pre-phosphorylated with CaMKII. As shown in Figure 2.7C, recombinant CaMKII phosphorylated bacterially-expressed Dronc at S130 (Figure 2.7C, lower panel), yet it had no effect on Dronc’s proteolytic activity toward its apoptosis-relevant substrate, drICE (Figure 2.7C, upper panel). These data indicate that phosphorylation of Dronc at S130 has no effect on its proteolytic activity once it is
dimerized and activated, and support that NADPH suppresses Dronc by inhibiting its activation rather than its catalytic activity.

2.3.2 Metabolic regulation of Dronc in transgenic animals

2.3.2.1 Elav-driven overexpression of UAS-Dronc results in caspase-dependent cell death in neuronal tissues

As S2 cells are an embryonic hemocyte-like cell line (Abrams et al., 1992), our observations imply that metabolic regulation of apoptosis likely functions in somatic cells as well as in oocytes. To examine this hypothesis in a whole organism, we first induced apoptosis by expressing UAS-WT Dronc constructs using different tissue-specific Gal4 drivers. When we used the eye-specific driver, GMR-Gal4, only homozygous animals (2× UAS-Dronc) exhibited eye ablation, the mosaic phenotype resulting from the death of eye pigment cells (see section 2.3.2.2). In contrast, overexpression of the UAS-Dronc constructs with the pan-neuronal driver, elav-Gal4, was able to cause inadequate wing expansion even in heterozygous animals (1× UAS-Dronc). Progeny exhibiting wing expansion defects could be categorized into three groups based on the severity of the phenotype, while their siblings carrying only the transgene or driver showed no morphological abnormalities (Figure 2.8A and see section 2.2.10). Moreover, newly-eclosed flies overexpressing WT Dronc (elav-Gal4>WT Dronc) remained untanned in their abdomen after incubation at 25°C for 3 h, whereas their siblings (driver only) exhibited normal maturation as evidenced by their darkened abdomen, indicating a delayed maturation (Figure 2.8B).
After eclosion, *Drosophila* wing expansion and sclerotization are known to be controlled by the neuropeptide hormone bursicon, which is produced by a subset of neurons named CCAP (Fraenkel & Hsiao, 1962; Luan et al, 2006). Overexpression of Reaper, a member of the RHG proteins, in CCAP neurons has also been shown to lead to defects in wing expansion and cuticle tanning (Dewey et al, 2004; Park et al, 2003), similarly to the defects we observed. To further characterize the defects induced by elav-driven overexpression of our UAS-Dronc constructs, another UAS construct encoding p35, a viral anti-apoptotic protein, was co-expressed in the flies. Notably, co-expression of p35 not only significantly restored bursicon levels in hemolymph, but also reduced the wing expansion defects regardless of the expression levels of Dronc (Figure 2.8C and D). These observations strongly suggest that the pathological phenotypes we observed are attributable to caspase-dependent bursicon deficiency, and thereby could be used as readout for Dronc’s activity in transgenic flies.

2.3.2.2 Metabolism suppresses death-inducing activity of Dronc in specific neuronal tissues, but not in the photoreceptor cells of the eyes

To further examine how NADPH levels control apoptosis in a whole organism, we artificially generated an energy crisis by expressing UAS-RNAi hairpins targeted against NADPH-generating enzymes. We then assessed how NADPH deficiency affected phenotypes resulting from overexpression of UAS-Dronc. As shown in Figure 2.9A, downregulation of malic enzyme (Men) aggravated wing expansion defects induced by the elav-driven overexpression of WT Dronc, indicating that NADPH
deficiency could enhance the death-inducing ability of WT Dronc in transgenic flies. This implied that the activity of WT Dronc might be suppressed by physiological levels of NADPH. In contrast, phosphomutant Dronc (S130A) appeared not to be sensitive to cellular NADPH levels, as coexpression of Men RNAi had no effect on the phenotype induced by overexpression of Dronc S130A, suggesting that NADPH suppressed Dronc in certain neuronal cells through metabolically-mediated phosphorylation of Dronc at S130. Indeed, when transgenic strains expressing similar levels of WT or non-phosphorylatable Dronc were compared, flies expressing the Dronc S130A phosphomutant exhibited far more severe wing expansion defects than those expressing WT Dronc, indicating that preventing the metabolically-induced inhibitory phosphorylation of Dronc could enhance its cell-killing activity in transgenic flies (Figure 2.9A and B). In contrast, GMR-driven overexpression of either WT or phosphomutant Dronc (S130A) caused similar eye phenotypes, suggesting that Dronc might not be regulated by NADPH in eye tissues (Figure 2.9C). Taken together, these data demonstrate that Dronc is metabolically-suppressed in neuronal tissues in vivo.

2.3.2.3 NADPH prevents unnecessary death during neuronal development in Drosophila through modulating Dronc

As Dronc is required for the development of several neuronal tissues in Drosophila (Choi et al., 2006; Koto et al., 2009; Kuo et al., 2006; Waldhuber et al., 2005), we then tested whether NADPH metabolism affected cell fate determination in these tissues. Surprisingly, downregulation of G6PDH, which has been shown to be
responsible for ~40% of NADPH production in *Drosophila* larvae (Geer et al., 1979; Merritt et al., 2009), through elav-driven expression of UAS-G6PDH RNAi resulted in severe wing expansion defects and delayed maturation in all progeny (Figure 2.9D), phenocopying elav-driven neuronal overexpression of UAS-Dronc (Figure 2.8A and B). This suggests NADPH deficiency during development can trigger apoptosis in specific neurons, possibly through activating Dronc.

**2.4 Discussion**

**2.4.1 NADPH levels set a threshold for *Drosophila* apoptosis**

DIAP1’s central role in regulating *Drosophila* apoptosis is well-recognized (reviewed by Hay and Gao, 2006 and Kumar, 2007); signals regulating apoptosis in flies are known to target either DIAP1 itself or its antagonists, the RHG proteins, which induce apoptosis by neutralizing DIAP1’s inhibition (Goyal et al., 2000; Hay and Guo, 2006; Kumar, 2007; Meier et al., 2000; Muro et al., 2002; Wang et al., 1999b; Wilson et al., 2002; Yoo et al., 2002). In the current study, using cultured S2 cells and transgenic animals, we revealed a novel regulatory circuit in *Drosophila* apoptosis wherein cellular metabolic status controls Dronc through CaMKII-mediated inhibitory phosphorylation of Dronc at S130 and thereby suppresses Dronc-mediated *Drosophila* apoptosis without directly modulating DIAP1.

Interestingly, endogenous Dronc appears to be subject to extensive post-translational modifications in S2 cells, as assessed by 2D-immunoblotting. Our Dronc
pS130 antibody recognizes several species of endogenous Dronc, suggesting that a considerable portion of endogenous Dronc is phosphorylated at S130 in healthy cells (data not shown). Based on our data, we suggest that when cell metabolism is compromised, NADPH deficits decrease the proportion of phosphorylated Dronc, facilitating the interaction between Dronc and Dark. Therefore, the threshold for apoptosis is lowered and cells are sensitized to apoptotic stimuli.

Notably, Dronc has been shown to function in a non-apoptotic context, mediating morphogenesis in Drosophila neuronal tissues (Koto et al., 2009; Kuo et al., 2006). However, little is known about how active caspases exert their nonapoptotic functions without triggering cell death. Current evidence suggests that limited activities of caspases may be sufficient to mediate cell fate decisions during neuronal differentiation without inducing cell death (Kanuka et al., 2005; Koto et al., 2009; Kuranaga et al., 2006). In support of this hypothesis, our results suggest that the cell-killing activity of Dronc can be modulated by cellular NADPH in vivo through its phosphorylation at S130, even in the absence of DIAP1. As DIAP1 is cleared from the cells during a particular developmental stage in neuronal tissues, such regulation would allow active Dronc to mediate neuronal differentiation without inducing unnecessary cell death.

2.4.2 Metabolic regulators are involved in caspase activation

Two studies using RNAi-based genome-wide silencing in Drosophila have been conducted by Yi et al., 2007 and Chew et al., 2009 to identify novel regulators of
Drosophila apoptosis. As we showed that dsRNA directed against malic enzyme (Men) and CaMKII regulates apoptosis, one may wonder why those factors were not identified in previous studies. This may be simply explained by the fact that both published reports identify factors protecting cells from apoptosis after their knockdown, while depletion of either Men or CaMKII would accelerate apoptosis. Blocking enzymes consuming NADPH, conversely, would be predicted to artificially increase cellular NADPH levels, resulting in protective effects on apoptosis. However, NADPH is utilized by multiple biological processes, so inhibition of a single pathway may not raise NADPH sufficiently to sustain a detectable protection upon prolonged apoptotic stimulation. Therefore, a genome-wide RNAi screen to identify genes required for malate’s protection from apoptosis could be fruitful in identifying novel apoptotic modulators.
Figure 2.1: Programmed cell death is metabolically regulated in Drosophila S2 cells.

Figure 2.1: (A-C) Malate overrides DHEA-induced apoptosis. (A-B) S2 cells were treated with different concentrations of DHEA ± 5 mM malate in glucose free medium for 24 h. After 24 h treatment, (A) photomicrographs were taken with a confocal microscope. (B) Treated cells were also incubated in 0.5μg/ml PI staining solution for 5 min and then the percentage of PI-positive cells was read by flow cytometry. Malate significantly suppressed DHEA-induced cell death in cells receiving 50 μM (**p < 0.01) or 100 μM DHEA (**p < 0.001). (C) Lysates from untreated S2 cells and cells treated with 100 μM DHEA ± 5 mM malate were analyzed for effector caspase-like activity using the caspase substrate Ac-DEVD-pNA. The addition of malate significantly reduced DHEA-induced caspase activity (**p < 0.001). (D) DHEA and malate modulates cellular NADPH levels. Lysates from cells receiving the same treatment as described above were analyzed for NADPH content. The DHEA-induced decrease in NADPH levels was significantly rectified by malate (**p < 0.001). Each bar in B-D represents the mean ± standard deviation (SD) from three independent experiments, and the p values were calculated according to two-tailed Student’s t test.
Figure 2.2: Dronc mediates apoptotic cell death induced by NADPH deprivation.

**Figure 2.2:** (A) Apoptosis induced by DHEA, DIAP1 dsRNA and CHX requires Dronc and Dark. S2 cells after 2 d pretreatment of 80 nM Dronc or Dark dsRNA were treated with 100 μM DHEA, 40 nM DIAP1 dsRNA or 40 μM CHX. Photomicrographs were taken 12 h (DIAP1 dsRNA and CHX) or 24 h (DHEA) post treatment. (B) Protein levels of Dronc and Dark after RNAi. Lysates from S2 cells treated with Dronc dsRNA were analyzed for endogenous Dronc using anti-Dronc antibodies. The efficacy of Dark RNAi was assessed as previously described (Zimmermann et al, 2002) (Dark1-411, Dark N-terminal truncate mutant containing CARD domain; asterisk, non-specific band). (C) DHEA requires Dronc to initiate the caspase cascade. The processing of endogenous Dronc and drICE in Dronc-deficient cells treated with 100 μM DHEA was examined by immunoblotting. Full-length and processed caspases are indicated by FL and P, respectively. (D) Malate blocks DHEA-induced Dronc activation. Lysates were collected at the indicated time points from S2 cells treated with 100 μM DHEA ± 5 mM malate in glucose free medium, and then analyzed for the cleavage of caspases by immunoblotting. The autoprocessing of Dronc and Dronc-mediated cleavage of drICE were used as readouts to monitor Dronc activation (FL, full-length; P, processed).
Figure 2.3: Metabolic status modulates Dronc activation.
Figure 2.3: (A-D) Malate inhibits spontaneous caspase activation induced by the loss of DIAP1. (A-B) Malate blocks spontaneous caspase activation induced by DIAP1 dsRNA. S2 cells were treated with 40 nM GFP or DIAP1 dsRNA ± 5 mM malate in serum-free Schneider’s medium for 12 h before pictures were taken (A) and lysates were collected for caspase assay (B). The addition of malate greatly suppressed an increase in caspase activity resulting from the loss of DIAP1 (**p < 0.01). (C-D) Malate suppresses CHX-induced apoptosis. S2 cells were treated with 40 μM CHX ± 5 mM malate in standard Schneider’s medium. (C) Pictures were taken 12 h post treatment. (D) Lysates from treated cells were also analyzed by caspase assay. CHX-induced caspase activation was significantly alleviated by the addition of malate (***p < 0.001). (E) Malate delays Dronc activation in CHX-treated cells. Lysates were collected at the indicated time points from S2 cells treated with 40 μM CHX ± 5 mM malate in standard Schneider’s medium, and then analyzed by immunoblotting. Spontaneous caspase activation was monitored by the autoprocessing of Dronc and Dronc-mediated cleavage of drICE (FL, full-length; P, processed). (F-G) Malate affects neither the expression levels nor the degradation of DIAP1. Lysates from S2 cells treated as indicated were analyzed by immunoblotting to determine (F) the expression levels or (G) the half-life of DIAP1. Actin blotting was used as loading control. (H) The Drosophila apoptotic pathway. Shaded boxes represent apoptotic inducers used in this study. Values represented in B and D are the means ± SD from three independent experiments. The p values were calculated according to two-tailed Student’s t test.
Figure 2.4: Men RNAi abrogates malate’s protection.

**Figure 2.4:** (A-B) Malate’s ability to inhibit CHX-induced apoptosis requires malic enzyme (Men). S2 cells pretreated with 80 nM (A) GFP or (B) Men dsRNA were then treated with 40 μM CHX ± 5 mM malate in standard Schneider’s medium. Photomicrographs were taken at different time points to monitor membrane blebbing and decreased cell density. (C) S2 cells ectopically expressing FLAG-tagged Men were treated with Men dsRNA to access the efficacy of Men RNAi (as endogenous Drosophila Men antibody was not available). To avoid variation between transfections, S2 cells were transfected together, and then split into 6 well plates for dsRNA treatments. Downregulation of FLAG-tagged Men was used as a proxy for the expression levels of endogenous Men. Actin blotting was used to ensure equal loading. (D) Men is required for malate’s protection against CHX-induced increase in caspase activity. Lysates from cells treated as described above were analyzed by caspase assay.
Figure 2.5: The death-inducing activity of Dronc is metabolically regulated through the inhibitory phosphorylation at S130.
Figure 2.5: (A) Metabolic status modulates S130 phosphorylation of ectopically-expressed Dronc. S2 cells expressing FLAG-tagged Dronc prodomain were labeled as described in section 2.2.5. Phosphorylation of WT and a panel of point-mutant Dronc prodomain were measured by autoradiography. Parallel immunoblotting was used to ensure equal loading. (B-C) Dronc pS130 antibody validation in S2 cells. (B) Dronc pS130 only recognized phosphorylated Dronc. FLAG-tagged full-length Dronc (C318A) was immunoprecipitated with FLAG beads, treated with λ-phosphatase, and then analyzed by immunoblotting using Dronc antibody and Dronc pS130 antibody. (C) Lysates from cells transfected with either Dronc C318A or Dronc C318A/S130A expression plasmids were examined by immunoblotting using Dronc antibody and Dronc pS130 antibody. (D) NADPH levels control the phosphorylation status of endogenous Dronc at S130. S2 cells were treated with 100 μM DHEA ± 5 mM malate in glucose-free medium supplemented with 50 μM zVAD for 6 h. Lysates from treated cells were collected for immunoblotting using Dronc antibody and Dronc pS130 antibody (Asterisk, non-specific band). (E) Stably transfected S2 cells (see section 2.2.4) were treated with CHX to induce apoptosis. Cells collected at different time points were used to assess the percentage of GFP-positive cells by FACS and then lysed to measure cellular DEVDase activity by caspase assay. (F) Mutation of Dronc S130 abolishes malate’s inhibition of cell death. S2 cells expressing sub-lethal levels of full-length WT Dronc or S130A phosphomutant were treated with 40 μM CHX ± 5 mM malate in standard Schneider’s medium for 8 h. The percentage of GFP-positive cells was examined by flow cytometry and used as an indicator of cell viability. Malate was able to rescue CHX-induced cell death in cells transfected with either empty vectors (***p < 0.001) or WT Dronc expression vectors (**p < 0.01), but did not protect cells expressing Dronc S130A phosphomutant (p = 0.544). Data were collected from three independent experiments, and shown as means ± SD. Two-tailed Student t tests were performed to determined p values. (G) Expression levels of WT Dronc or S130A phosphomutant C-terminally tagged with protein A in stable lines were examined by immunoblotting using anti-protein A antibodies, and actin was used as a loading control.
Figure 2.6: CaMKII phosphorylates Dronc S130 \textit{in vivo} and \textit{in vitro}.

\textbf{Figure 2.6:} (A) Neither PKA nor CK1 inhibitors abrogates malate’s protection. S2 cells pretreated with PKA (30 μM H-89) (Zhao et al., 2007) or CK1 (8 μM IC261) (Mennella et al., 2009) inhibitors were treated with 40 μM CHX ± 5 mM malate in standard Schneider’s medium. Cell density and viability were recorded by pictures. (B) CaMKII RNAi abrogates malate’s inhibition. S2 cells pretreated with GFP, PKA, CK1, CaMKI or CaMKII dsRNA were then treated with 40 μM CHX ± 5 mM malate in regular Schneider’s medium. Cell death and membrane blebbing were recorded 12 h post CHX treatment. (C) CaMKII is required for phosphorylation of Dronc at S130 \textit{in vivo}. CaMKII-deficient cells were treated with 5 mM malate in glucose free medium as indicated. Lysates were examined by immunoblotting for the phosphorylation status of endogenous Dronc at S130. (D) CaMKII phosphorylates the Dronc prodomain at S130 \textit{in vitro}. GST-tagged recombinant Dronc prodomain (WT, S127A and S130A) and GST were incubated with CaMKII and 32P-ATP in kinase buffer as described in section 2.2.7. Sample amounts and phosphorylation status were examined by coomassie blue and autoradiography respectively.
Figure 2.7: Phosphorylation at Dronc S130 prevents its interaction with Dark but has no effect on its catalytic activity.

**Figure 2.7:** (A) Phosphorylation at Dronc S130 dampens the interaction between Dark and Dronc. Lysates containing HA-tagged Dronc molecules and FLAG-tagged Dark’s CARD domain (Dark1-411) were incubated with FLAG beads. Precipitates were analyzed by immunoblotting with FLAG antibodies to show that the same amount of Dark was precipitated, and with HA antibodies for Dronc molecules interacting with Dark’s CARD. Expression levels and phosphorylation status of ectopically-expressed proteins in lysates were also assessed by immunoblotting, where an actin blotting was used to verify equal loading. Shown are representative data from three independent experiments (asterisk, non-specific band). (B) Similar experiment was performed as described in A to examine the interaction between DIAP1 and Dronc. (C) Phosphorylation at Dronc S130 has no effect on its catalytic activity. *In vitro* translated drICE was incubated with mock-treated or CaMKII-treated recombinant Dronc at 25°C with occasional shaking for 2 h. Dronc-mediated processing of drICE was monitored by autoradiography. Protein levels and phosphorylation status of Dronc were examined by immunoblotting using the antibodies indicated.
Figure 2.8: Overexpression of Dronc causes apoptosis in neuronal cells

Figure 2.8: (A) Overexpression of WT Dronc in neuronal tissues through elav-Gal4 induces wing expansion defects. Flies expressing WT Dronc were categorized into three groups (elav-Gal4/+; +; UAS-Dronc/+). Same-sex siblings carrying driver or transgene only were also shown as control (elav-Gal4/+; +; + and +; +; UAS-Dronc/+; +). Progeny from different crosses were categorized and scored based on their phenotypes as described in section 2.2.10. (B) Photograph taken 3 h after eclosion of female age-matched elav-Gal4>UAS-WT Dronc (left) or sibling carrying driver only (right) shows the inhibition of cuticle tanning in animals ectopically-expressing WT Dronc in neuronal tissues. (C-D) Coexpression of p35 suppresses Dronc-induced bursicon deficiency in hemolymph and the consequent wing expansion defects, where W (Weak) and S (Strong) indicates the expression levels of Dronc. (C) Bar graph shows the frequency of wing expansion deficits in the progeny The Chi-square analysis shows that defects induced by WT Dronc were significantly suppressed by p35 (***p < 0.001). Genotype: elav-Gal4/+; +; UAS-p35/UAS-WT Dronc. (D) Immunoblot analysis shows bursicon levels in hemolymph extracted from age and sex matched newly-eclosed progeny.
Figure 2.9: NADPH metabolism regulates Dronc-mediated apoptosis in vivo.
Figure 2.9: (A) Cell-killing activity of Dronc is metabolically-suppressed in transgenic flies through phosphorylation of Dronc at S130. Indicated lines were crossed with elav-Gal4. Data were analyzed by Chi-square test (**p < 0.01, ***p < 0.001, N.S., not significant). Genotype: elav-Gal4/+; +; UAS-Dronc/+ and elav-Gal4/+; UAS-MEN RNAi hairpins +; UAS-Dronc/+.

(B) Expression levels of Dronc. Lysates from 10 newly-eclosed progeny with desired genotype were analyzed by immunoblotting using anti-FLAG antibodies for FLAG-tagged WT Dronc or S130A phosphomutant (C) Overexpression of WT Dronc or Dronc S130A phosphomutant results in similar eye defects. Two lines strongly expressing either WT Dronc (WTΔ) or Dronc S130A phosphomutant (S130A5) were crossed with DIAP1-deficient flies (Df(3L)st-f13) or flies constitutively expressing rpr in the eye tissues (GMR-rpr) at 18°C to avoid unnecessary death. Crosses using flies carrying only Dronc transgenes (1× and 2× Dronc) were incubated at 23°C instead to enhance eye phenotype. Expression of transgenes as indicated was driven by GMR-Gal4.

Genotype: Driver only (+; GMR-Gal4/+; TM3/+; +), 1× Dronc (+; GMR-Gal4/+; UAS-Dronc/TM6B; +), 1× Dronc + ΔDIAP1 (+; GMR-Gal4/+; UAS-Dronc/Df(3L)st-f13; +), 1× Dronc + GMR-rpr (+; GMR-Gal4/+; UAS-Dronc/GMR-rpr; +), 2× Dronc (+; GMR-Gal4/+; UAS-Dronc/UAS-Dronc; +). (D) Downregulation of G6PDH in neuronal tissues causes delay in maturation and wing expansion defects. Pictures were taken 3 h (upper panel) or 24 h (lower panels) post eclosure. Genotype: elav-Gal4/+; UAS-G6PDH RNAi hairpins/+; + and elav-Gal4/+; +; +. Shown are sex- and age-matched progeny from crosses set up at 23°C.
Chapter 3  Identification of signaling pathways underlying the metabolic control of initiator caspases

3.1 Introduction

The data presented in Chapter 2 demonstrated that NADPH-mediated control of caspase activity applies not only to germ cells, as might be concluded based on the regulation of caspase-2 in oocytes (Nutt et al., 2005), but also to somatic cells, as both hemocyte-like S2 and elav-expressing neuronal cells were subjected to metabolic control. These findings suggest that initiator caspases, such as caspase-2 in vertebrates and Dronc in flies, are suppressed by nutrients in most tissues through constitutive phosphorylation. Meanwhile, in a parallel project using cultured human cancer cells and mouse oocytes, our laboratory demonstrated that Dronc and caspase-2 (in mammals and Xenopus), are suppressed via inhibitory phosphorylation mediated by NADPH (at S130, S164, and S135, respectively) (Nutt et al., 2009; Yang et al., 2010). These observations suggest that the signaling pathway underlying this metabolic regulation is well conserved throughout evolution.

Of note, most cancer cells exhibit altered metabolism, where NADPH has been found to be robustly produced from enhanced aerobic glycolysis (which stimulates the PPP flux) and glutaminolysis (which accelerates a part of TCA cycle to produce more malate that is then exported out of mitochondria and converted into NADPH and pyruvate) (Levine and Puzio-Kuter, 2010). Based on our observations, abnormally sustained levels of cellular NADPH may suppress initiator caspases, and thereby allow
cancer cells to escape from apoptosis, one of the well recognized hallmarks of cancer (Hanahan and Weinberg, 2011). In Chapter 3, we attempt to further elucidate the signaling pathway linking NADPH metabolism and the activation of initiator caspases. This may lead to a better understanding of cancer pathology and potentially help identify novel therapeutic targets.

To this end, we employed both targeted and unbiased approaches: tracking the metabolic fate of NADPH and designing a whole genome screen based on bimolecular fluorescence complementation (BiFC) that directly monitors Dronc activation. Our data suggest that fatty acid synthase (FASN) plays a crucial role in NADPH-mediated suppression of Dronc and caspase-2. Abrogation of this metabolic suppression with an FASN inhibitor triggered caspase-2 dependent cell death in various ovarian cancer cells. In the second part, we validated the Dronc BiFC system by studying the kinetics of its fluorescence signals and demonstrated its sensitivity to a variety of apoptotic inducers. Data presented in this Chapter are preliminary and more detailed analyses are currently underway.

3.2 Materials and methods

3.2.1 Drosophila S2 cell culture and viability test

_Drosophila_ cell culture, viability test, and dsRNA synthesis are described in section 2.2, except for the Dronc BiFC related experiments (see below).
3.2.2 Construction of Dronc BiFC plasmids

DNA encoding the prodomain region of Dronc (1-135) was inserted into pBiFC-VN173 and pBiFC-VC155 (both vectors were purchased from Addgene). The open reading frame (ORF) containing a 1×FLAG tag, Dronc prodomain, VN173 fragment, and a stop codon was selectively amplified by PCR and then inserted into pMK33-CTAP (a kind gift from Dr. David MacAlpine at Duke University) under a metallothionein promoter (no TAP tag could be expressed due to the inserted stop codon); purified ORF containing a 1×HA tag, Dronc prodomain, VC155 fragment and a stop codon was inserted into pMT-puro (Addgene). Maps of the final constructs are shown below. The backbones for pHygroBiFC Dronc-VN173 and pPuroBiFC Dronc-VC155 are pMK33-CTAP and pMT-puro, respectively.

Figure 3.1: Maps of Dronc Bimolecular fluorescence complementation constructs
3.2.3 Bimolecular fluorescence complementation

*Drosophila* S2 cells were transfected in a T25 flask with 0.5 μg of pHygroBiFC-Dronc-VN173 and 0.05 μg of pPuroBiFC-Dronc-VC155 by Effectene (QIAGEN) according to the manufacturer’s instructions. Transfected cells were selected in standard Schneider’s medium supplemented with 125 μg/ml hygromycin B (Invitrogen) and 1 μg/ml puromycin (Invitrogen) for more than 3 weeks. For BiFC experiments, stably transfected cells were induced with 100 μM CuSO₄ for 16 h and then cultured in fresh regular Schneider’s medium for another 24 h. After induction, cells were treated with 40 μM CHX, 50 μM etoposide, 50 μM taxol, or 50 μM C75 (indicated in the figure legends) with 50 μM pan-caspase inhibitor zVAD to prevent cell death (except for those cells to be assayed for caspase activities). Effector-like caspase activity and fluorescence intensity were then measured by caspase assay and flow cytometry as previously described in section 2.2. Although the mean fluorescence values fluctuated between experiments, the same trend is conserved. Bar graphs indicate representative mean fluorescence intensity values from at least three independent experiments.

3.2.4 Mammalian cell culture

Human ovarian cancer cells OVCA420 and OVCA432 (hereafter called 420 and 432, respectively) were kind gifts from Dr. Susan Murphy (Duke University) and maintained in RPMI1640 with 10% FBS; human ovarian cancer cells ES-2 (#CRL-1978) were purchased from American Type Culture Collection (ATCC) and maintained in McCoy’s 5A with 10% FBS. Cells were transfected with FuGENE 6 (Promega) and
Lipofectamine RNAiMAX (Invitrogen) for transient and siRNA transfections, respectively. Caspase-2 3’-UTR siRNA (UGGAAGUAUUUGAGAGAGAUU) was transfected at 10 nM (final concentration). Transfected cells were collected for cell lysate or analyzed for PI-positive cells by flow cytometry 72 h post transfection, as previously described in section 2.2. For orlistat treatment, cells were switched into fresh culture medium supplemented with 50-300 μM orlistat or the same volume of solvent (DMSO) for 24 h before further analyses.

### 3.2.5 Immunoblotting

Treated cells were lysed in cell lysis buffer (150 mM Tris, pH 7.7, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 1× Roche complete protease inhibitor, 10 mM Na₄P₂O₇). Protein concentration was measured by Bradford assay with spectrophotometry. 30-100 μg of cell lysate resolved by SDS-PAGE was transferred to a PVDF membrane, and blocked for 30 min at room temperature in 1X PBS with Tween-20 (Bio-Rad) and 5% skim milk or BSA (Sigma). Membranes were incubated with primary antibody at 4°C overnight. Afterward, membranes were washed four times in 1X PBS with Tween-20, and then incubated with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (Promega, 1:5,000) for 1 h at room temperature, followed by the same wash as described above. For detection, an enhanced chemiluminescence substrate solution (Western Lightning ECL, Perkin Elmer) was added to the immunoblots, and a film was used to detect the signals. Rabbit anti-caspase-2 (Abcam,
1:1,000), rabbit anti-actin (Santa Cruz, 1:10,000), and rabbit anti-Bid (Cell signaling, 1:1,000) were utilized in this part of study.

### 3.3 Results

#### 3.3.1 Fatty acid synthase (FASN) is involved in NADPH-mediated metabolic regulation of caspases in S2 and ovarian cancer cells

##### 3.3.1.1 NADPH-mediated suppression of *Drosophila* apoptosis requires FASN

To further elucidate the players responsible for the NADPH-mediated suppression of caspases, we first examined the involvement of fatty acid synthase (FASN), which catalyzes one of the major cellular NADPH-consuming reactions. Consistent with our previous observations, malate protected *Drosophila* S2 cells from CHX-induced apoptosis; however, orlistat, a novel chemical inhibitor of FASN (Kridel et al., 2004), abrogated malate’s protection, as in the presence of orlistat, CHX treatment was able to cause the loss of cell density even in the presence of malate (Figure 3.2A).

We also used dsRNA to specifically downregulate cellular FASN activity. As shown in Figure 3.2B, in the presence of malate, FASN-deficient cells were still sensitive to CHX. In contrast, when cellular NADPH levels were raised by malate addition, CHX-induced apoptosis was significantly mitigated in cells pretreated with GFP or CaMKI dsRNA. These two observations, collectively, demonstrate an essential role of FASN for NADPH-mediated suppression of *Drosophila* apoptosis.
3.3.1.2 Inhibition of FASN activity triggers cell death and caspase-2 activation in ovarian cancer cells

Interestingly, abnormally enhanced levels of FASN have been broadly observed in human cancers of various origins, such as ovarian, breast, prostate, thyroid, renal and neuronal cells (Kuhajda, 2006). Inhibition of FASN exhibits selective cytotoxicity to cancer cells in vivo and in vitro, at least in part due to apoptotic cell death (Bandyopadhyay et al., 2006; Brusselmans et al., 2003; Carvalho et al., 2008; De Schrijver et al., 2003; Knowles et al., 2008; Lee et al., 2009; Zecchin et al., 2011). However, the precise mechanisms utilized are still unclear. As our data suggested that blocking FASN would facilitate the activation of initiator caspase by removing an inhibitory phosphorylation, we then examined the potential role of caspase-2 (the vertebrate initiator caspase subjected to NADPH-mediated metabolic suppression) in apoptosis triggered by FASN inhibition.

Similarly to published reports, we observed massive cell death after blocking FASN activity with orlistat in many human ovarian cancer cell lines, including ES-2, 432, and 420 (Figure 3.3A). To examine whether caspase-2 was involved, we monitored the cleavage of caspase-2 and its downstream target Bid, which could be used as an indicator for their pro-apoptotic activities. In support of our hypothesis, full-length caspase-2 and Bid were significantly decreased upon orlistat treatment, indicating that these two pro-apoptotic proteins were activated after FASN inhibition, likely contributing to the subsequent apoptosis (Figure 3.3B).
3.3.1.3 FASN inhibition removes NADPH-mediated inhibitory phosphorylation of caspase-2

In mammals, caspase-2 is suppressed by an inhibitory phosphorylation at S164 in response to abundant NADPH. This site is located in the prodomain, and is potentially capable of preventing caspase-2 from interacting with its activating adaptor protein, RAIDD (Nutt et al., 2009; Nutt et al., 2005). Since the data presented in Figure 3.2 demonstrate that orlistat abrogates NADPH-mediated suppression of apoptosis, potentially though blocking its ability to maintain an inhibitory phosphorylation of Dronc, we then examined how orlistat modulated NADPH-regulated phosphorylation of caspase-2.

To this end, ES-2 cells were transfected to express Myc-tagged human caspase-2 prodomain to enhance detection. Total and phosphorylated populations were detected by Myc antibody and a phospho-specific antibody targeted against phosphorylated S164 of human caspase-2 (hCasp2 pS164). As shown in Figure 3.4A, caspase-2 S164 appeared to be phosphorylated in untreated cells, suggesting that at least some population of caspase-2 was phosphorylated and inactive in healthy cells harboring normal NADPH metabolism. In contrast, once FASN activity was compromised by orlistat, the inhibitory phosphorylation at S164 was lifted, permitting caspase-2 activation.

3.3.1.4 Cell death induced by FASN inhibition requires caspase-2

To further confirm the role of caspase-2 in cell death resulting from FASN suppression, we specifically reduced caspase-2 expression levels with small interfering RNA (siRNA) targeted against the 3’ UTR region of caspase-2 transcripts (see section 3.2
for details), and examined how this reduction affected orlistat-induced cell death. We first monitored the cleavage of caspase-2 and Bid upon orlistat treatment by immunoblotting. Interestingly, downregulation of caspase-2 potently inhibited Bid cleavage upon orlistat treatment, demonstrating that the death signal along this axis was greatly impaired in the absence of caspase-2 (Figure 3.4B).

Lastly, we used flow cytometry to analyze cell death triggered by orlistat in cells receiving either control or caspase-2 siRNA. Strikingly, suppression of caspase-2 activity by siRNA greatly prevented orlistat-induced cell death in ES-2 cells, indicating that the activation and proteolytic activity of caspase-2 were indispensable for cell death resulting from FASN inhibition (Figure 3.4C).

In summary, the data presented in section 3.3.1 collectively suggest that FASN activity is required for NADPH-mediated regulation of initiator caspases. Moreover, we further clarified the molecular mechanisms of cell death induced by FASN inhibition by demonstrating caspase-2’s involvement. Future experiments will be directed towards determining precisely how FASN modulates both Dronc and caspase 2 upstream of cell death.

3.3.2 Monitoring Dronc activation by an assay based on bimolecular fluorescence complementation (BiFC)

3.3.2.1 Construction and expression of the Dronc BiFC system

To globally identify regulators involved in the metabolic control of Dronc, we designed an assay based on the BiFC technique, which has been widely used to study
protein-protein interactions. A recent report from the laboratory of Dr. Douglas Green utilized this technique to monitor the dimerization of the caspase-2 prodomain, and identified Hsp90 as a modulator of caspase-2 activation (Bouchier-Hayes et al., 2009). As the feasibility of using BiFC to monitor caspase-2 activation was verified in this study, we then tried to design a similar assay to monitor Dronc activation in Drosophila S2 cells.

To accomplish this, we constructed plasmids encoding the Dronc prodomain fused to either N- or C-terminal Venus fragments. The truncated Venus fragments emit fluorescence only when juxtaposed (e.g., Dronc prodomains dimerize in response to apoptotic stimuli). Therefore, BiFC signals can serve as a real-time indicator, allowing us to monitor Dronc activation in a high-throughput system. Together with the Drosophila whole genome RNAi library, we can perform a loss-of-function screen for genes that are required for cell death induced by different apoptotic stimulus (Blocking the induction of BiFC when knockdowned) or for genes that are essential for malate’s protection (restoring malate-mediated suppression of BiFC when knockdowned).

Antibiotic resistance genes on the plasmids permitted the selection of stably transfected cells (please see section 3.2.2 for details on plasmid construction and stable line selection). Selected cells could be induced by CuSO₄ to express the Dronc BiFC fusion molecules, which are depicted and shown in Figure 3.5A. To verify the expression of both molecules, lysates from mock- and copper-treated cells were analyzed by immunoblotting with FLAG and HA antibodies. As shown in Figure 3.5B, stably transfected cells expressed both molecules after induction. We then examined whether
this system could emit fluorescence in response to apoptotic stimuli. Although some cells showed a basal level of fluorescence after induction, the canonical apoptosis inducer, CHX, stimulated fluorescence emission from many more cells (Figure 3.5C, right panel). To quantify the difference, we measured the mean fluorescence intensity in mock- or CHX-treated cells by flow cytometry. Consistent with our previous observations, CHX strongly elevated the mean fluorescence intensity by 4.5 fold (Figure 3.5D), demonstrating that fluorescence intensity correlated with the presence of apoptotic stimuli.

3.3.2.2 Kinetics of BiFC signals

We also examined the kinetics of the Dronc BiFC signals in response to CHX. To this end, CuSO4-induced cells were divided into two parts: one used to measure effector-like caspase activity, and the other used to detect Dronc BiFC signals (please see the figure legends and section 3.2 for detailed procedures). As shown in Figure 3.6, Dronc BiFC readily responded to CHX within 1 h post treatment (Figure 3.6, right panel); by contrast, effector-like (DEVDase) activities were not raised till 2.5 h after CHX addition (Figure 3.6, left panel), consistent with the idea that Dronc BiFC signals reflect the initial step of the caspase cascade.

3.3.2.3 Dronc BiFC can be induced by various apoptotic stimuli

Given the ability of CHX to induce fluorescence in our Dronc BiFC system, we next attempted to extend this finding by challenging the system with a variety of
apoptotic stimuli. Several pro-apoptotic reagents have been shown to induce cell death through Dronc, including DNA damage inducers (e.g., etoposide) and microtubule modulators (e.g., taxol). We then examined the ability of these reagents to stimulate fluorescence signals in our system. As shown in Figure 3.7, both etoposide and taxol induced considerable fluorescence 24 h post treatment. Additionally, given that our previous data suggested FASN could also modulate Dronc activation, we treated cells with the FASN inhibitor, C75, to measure its impact on BiFC fluorescence. Indeed, inhibition of FASN activity by C75 elicited a strong fluorescence response, similar to that induced by CHX. Again, this observation further confirms that cellular FASN activity regulates cell death by modulating the activation of the initiator caspase Dronc (Figure 3.7).

Overall, our data suggest that the fluorescence signal from our Dronc BiFC system can be used as an indicator of Dronc activation. Thus, this system, with the aid of the *Drosophila* whole genome RNAi library, will enable us to perform unbiased screens to identify novel apoptotic regulators that control apoptosis resulting from different stresses.

### 3.4 Discussion

#### 3.4.1 FASN is an essential regulator for the metabolic control of initiator caspases

In this Chapter, we further pursued the molecular mechanism underlying the metabolic control of Dronc. Since NADPH production has been shown to be essential for this metabolic control, we first analyzed its metabolic fate with the hope of discovering the missing link. In fact, NADPH, as one of the most important reductive metabolites in
the cell, has been shown to be actively involved in numerous biological processes, including the regulation of redox homeostasis and the biosynthesis of cellular components. NADPH is required for the conversion of glutathione disulfide (GSSG) back to reduced glutathione (GSH). Since GSSG and GSH are important redox compounds that neutralize most of the reactive oxygen species (ROS) in the cell, reduced NADPH levels may cause cell death secondary to redox imbalance. However, neither promotion nor suppression of GSH production (by adding N-acetylcysteine or L-buthionine-sulfoximine, respectively) affected malate’s protective effects against CHX-induced apoptosis in S2 cells (data not shown). Therefore, NADPH-mediated suppression of Dronc is unlikely due to changes in cellular redox status.

Intriguingly, our data suggested a crucial role for FASN activity in the metabolic regulation of Dronc. Blockade of FASN by orlistat abrogated malate’s protection, resensitizing S2 cells to CHX-induced apoptosis despite the presence of abundant NADPH. Orlistat treatment even directly activated caspase-2 in human ovarian cancer cells, resulting in Bid cleavage and subsequent cell death. Most importantly, phosphorylation at caspase-2 S164, which has been shown to be regulated by NADPH in a parallel study, was decreased upon FASN inhibition, supporting the notion that FASN is a key component in the NADPH-mediated inhibition of initiator caspases.

3.4.2 The Dronc BiFC system as an indicator of Dronc activation

As described in Chapter 1, caspase activation is tightly controlled by multiple regulatory systems. Oftentimes, the complexity of this regulatory network hinders the
discovery and characterization of novel apoptotic regulators. To understand how NADPH regulates Dronc activation, we designed the Dronc BiFC system to directly monitor Dronc dimerization. This system allows us to bypass downstream signaling and monitor the initial step of the *Drosophila* caspase cascade. To overcome the low transfection efficiency typical of S2 cells, we selected stably transfected cells that inducibly express two types of chimera molecules (a Dronc prodomain fused with an N- or C-terminal truncated Venus fragment) as assessed by immunoblotting. A fluorescent signal is emitted when the complementary Venus fragments come in close contact with each other, as exemplified with Dronc prodomain dimerization. Indeed, we observed enhanced fluorescence following treatments with several pro-apoptotic reagents of different mechanisms, validating this system as an indicator of caspase activation. Moreover, Dronc BiFC signals were detectable prior to effector-like caspase activity, further showing the sensitivity of this system to detect the death message at an early stage.

Although a similar system to monitor caspase-2 activation has been established in mammalian cells, modifications of the system are required to detect the activation of fly caspases with the BiFC assay, due to the distinct mode of caspase regulation in *Drosophila*. In healthy fly cells, dimerization of initiator caspases still occurs. Indeed, we observed low albeit consistent levels of fluorescence in induced cells even in the absence of apoptotic stimuli. To reduce this “background”, induced cells are cultured in fresh medium (without copper) for an additional 24 h before being used for experiments. Why this extra incubation step reduces background noise is unclear. We speculated that the
Additional incubation may allow endogenous DIAP1 to bind to the newly-synthesized Dronc BiFC molecules that otherwise readily dimerize. Using this approach, we were able to spread the differences in signal levels between mock- and CHX-treated cells. However, we still faced a substantial dilemma: inducing expression with lower concentrations of copper resulted in larger fluctuations in fluorescence between experiments; however, if we increased the copper concentration or prolonged the induction time, the background signals increased, and eventually masked the difference between pre- and post-CHX treatments, probably due to the exhaustion of the endogenous DIAP1 pool. The Dronc BiFC-related data presented above are representative of at least three independent experiments. We are currently testing additional protocols to get an optimal condition necessary for producing stable results.
Figure 3.2: Fatty acid synthase (FASN) activity is required for NADPH-mediated suppression of apoptosis in *Drosophila* S2 cells.

**Figure 3.2:** (A) Orlistat abrogates NADPH-mediated protective effects against CHX-induced apoptosis. S2 cells were treated as indicated with 40 μM CHX, 5 mM malate, and/or 50 μM orlistat in regular Schneider’s medium. Cell death and membrane blebbing were recorded 12 h post treatment. (B) FASN is required for NADPH-mediated suppression of CHX-induced apoptosis. Cells pretreated with different dsRNA were treated with 40 μM CHX ± 5 mM malate for 12 h and then examined for the loss of cell density.
Figure 3.3: Blocking FASN induces apoptotic cell death and caspase-2 activation in human ovarian cancer cells.

**Figure 3.3:** (A) Orlistat causes cell death in human ovarian cancer cells. Cells treated with 300 μM orlistat or the same volume of DMSO were analyzed by flow cytometry for dead cells (PI-positive cells) 24 h post treatment (B) Orlistat induces the cleavage/activation of caspase-2 and its downstream target, Bid. Lysate from cells treated as described was blotted for full-length caspase-2 and Bid. Actin was monitored as a loading control.
**Figure 3.4:** Caspase-2 is involved in apoptosis resulting from FASN inhibition.

**Figure 3.4:** (A) Caspase-2 prodomain is hypophosphorylated upon orlistat treatment. Cells ectopically expressing Myc-tagged human caspase-2 prodomain were treated with 300 μM orlistat for 24 h and then lysed for immunoblotting with Myc antibody and a phospho-specific antibody targeted against phosphorylated S164. (B) Caspase-2 siRNA blocks orlistat-induced Bid cleavage. Cells transfected with control or caspase-2 (hCasp2) siRNA were treated with 300 μM orlistat for 24 h. Full-length caspase-2 and Bid were then examined by immunoblotting with actin as a loading control. (C) Orlistat-induced cell death is significantly alleviated by caspase-2 siRNA. Cells treated as described in B were analyzed by flow cytometry for dead/PI-positive cells.
Figure 3.5: Construction and expression of Dronc BiFC.

Figure 3.5: (A) Schematic diagram depicts both Dronc BiFC molecules, consisting of a truncated Venus fragment (VN173, N-terminal fragment 1-173 or VC155, C-terminal fragment 155-238), Dronc prodomain and 1×FLAG or 1×HA tag. The truncated Venus fragments emit fluorescence only when juxtaposed (e.g., Dronc prodomains dimerize in response to apoptotic stimuli. (B) The expression of both Dronc BiFC molecules was induced with copper in stably transfected cell. Immunoblotting demonstrated the expression levels of the two molecules are comparable. Actin blot is used as a loading control. (C) Fluorescence is stimulated in cells expressing the Dronc BiFC molecules by a pro-apoptotic reagent, CHX, 8 h post treatments. Cell death was prevented by 50 μM pan-caspase inhibitor zVAD. The same randomly picked field was recorded by phase-contrast microscopy (upper) and fluorescence microscopy (lower). (D) Cells treated as indicated were analyzed by flow cytometry for the mean of fluorescence intensity. Reprehensive data from four independent experiments are shown.
Figure 3.6: Dronc BiFC signal Kinetics during apoptosis.

Figure 3.6: Cells were induced with copper to express the Dronc BiFC molecules, evenly split into two groups, and treated as indicated (CHX, 40 μM; zVAD, 50 μM). Treated cells were collected every 0.5 h for 4 h to assay for effector-like caspase (DEVDase) activity by a caspase assay and flow cytometry for fluorescence intensity. The caspase assay began detecting signals above the background noise level from lysates collected after 2.5 h post treatment (left panel); whereas Dronc BiFC was able to sense the death signal as early as 1 h post treatment (right panel).
Figure 3.7: Dronc BiFC detects caspase activation induced by various stimuli.

Figure 3.7: Cells treated as described in section 3.2 were examined by flow cytometry for mean fluorescence intensity. Four pro-apoptotic reagents were used, all of which stimulated different levels of fluorescence 24 h post treatment.
Chapter 4  Conclusion and Perspectives

Although DIAP1’s central role in regulating *Drosophila* apoptosis has been well recognized, several recently published results from genetic and whole genome RNAi screens suggest an extra layer of regulation that acts directly on caspases. Here, by studying the links between cellular metabolism and apoptosis, we revealed a novel signaling circuit that controls fly apoptosis in the post-DIAP1 phase. As discussed in Chapter 1, caspases appear to be controlled by several types of post-translational modifications. However, the regulatory roles of most caspase modifications have only been characterized in mammals. For example, although the phosphorylation of mammalian caspases has drawn a lot attention, it is surprising to find that Dronc S130, the metabolism-regulated phosphorylation site we identified in Chapter 2, was the first phosphorylation site ever to be characterized on a *Drosophila* caspase. Thus, data presented in this dissertation not only reveal a novel DIAP1-independent regulation of *Drosophila* apoptosis, but also further our understanding of caspase regulation in the fly.

In Chapter 3, we shed light upon the upstream signaling pathway underlying this novel regulation and extended our scope across species. The indicator system for Dronc activation developed in this dissertation to detect Dronc activation, together with established whole genome RNAi screening techniques in *Drosophila*, will allow us to identify additional players essential for the metabolic regulation of apoptosis in future investigations.
4.1 Metabolic control of initiator caspases by prodomain phosphorylation

Both Dronc and caspase-2 are initiator caspases, exerting their activity at the apex of the caspase cascades to control the fate of Drosophila and vertebrate cells. Although their functional niches appear to be different, as Dronc directly activates its downstream effector caspases, while caspase-2 cleaves a pro-apoptotic Bcl-2 family member, Bid, to control cytochrome c release, the data presented in Chapter 2 uncover an intriguing similarity in their regulation. Dronc and Xenopus caspase-2 are both phosphorylated by CaMKII in response to cellular NADPH levels (at S130 and S164, respectively). Through this mechanism, cellular NADPH metabolism prevents these caspases from interacting with their activating adaptor proteins, dampening apoptotic cell death.

In both cases, NADPH-regulated phosphorylation is located in the prodomain, which is the region responsible for the interaction with their regulators, such as DIAP1 and Dark, two important regulators of Dronc; thus, phosphorylation that occurs in the prodomain of initiator caspases may modulate these important regulatory interactions. Based on an in vivo labeling assay, we identified S130 as the primary (and possibly the only) phosphorylation site on the Dronc prodomain. This site is downstream of the CARD domain, and located in a linker that bridges the prodomain and the large catalytic domain. Notably, phosphorylation at Dronc S130 seems to prevent only Dark, but not DIAP1, from binding to Dronc. Indeed, it has been shown that only mutations in amino acids 118-122 of Dronc can affect DIAP1’s binding (Chai et al., 2003). So, how does this phosphorylation affect Dronc activation? Steric hindrance seems to be the most
straight forward explanation. Unfortunately, the only Dronc crystal structure currently available was generated using a truncated protein lacking the prodomain (Yan et al., 2006), and thus provides no clue about possible mechanisms. Alternatively, phosphorylation of Dronc S130 may serve as a docking site to recruit phospho-binding proteins, thereby masking Dark’s binding site on Dronc. In the case of Xenopus caspase-2, NADPH-mediated phosphorylation at S135 recruits 14-3-3, which in turn protects this site from dephosphorylation by PP1 and may also prevent caspase-2 from binding to its activating protein, RAIDD. In Chapter 2, we identified CaMKII as the relevant kinase responsible for the phosphorylation of Dronc S130. Further examinations of how PP1 and 14-3-3 are involved in the metabolic regulation of Dronc are of great interest.

4.2 NADPH metabolism suppresses Dronc in specific neuronal cells but not in the eye

In addition to cultured cells, in Chapter 2 we also examined how NADPH metabolism modulates Dronc in transgenic flies, which validated our findings in a more physiological context. Powered by the UAS-Gal4 system, we overexpressed wild-type and phosphomutant (S130A) Dronc in different tissues to examine how S130 phosphorylation modulated its killing activity. Our data obtained from S2 cells suggests that wild-type Dronc (if not all, at least a portion) is readily phosphorylated once expressed, whereas the Dronc S130A mutant evades metabolic suppression and exhibits enhanced cell-killing ability. Indeed, we observed that overexpression of the Dronc S130A mutant triggers much more severe neuronal cell death than that caused by wild-
type Dronc. In contrast, the eye ablation resulting from both forms of Dronc is indistinguishable, suggesting the NADPH metabolism may not be able to suppress wild-type Dronc in the eye.

Since the molecular mechanisms accounting for the inhibitory effects of Dronc S130 phosphorylation are still under investigation, it might be too early to speculate about the reason for this inter-tissue discrepancy. Nevertheless, the expression profile of *Drosophila* CaMKII in different tissues may provide a hint. Interestingly, CaMKII, the kinase acting on Dronc S130, is highly and predominantly expressed in neuronal tissues. In *Drosophila* adults, the CaMKII expression level in the brain is ~30 times higher than that of the eye. Therefore, we speculate that the relative insensitivity of Dronc to metabolic regulation in the eye may simply reflect a lack of CaMKII required to transduce the metabolic signal.

Interestingly, the proteolytic activity of Dronc has been shown to play a crucial “non-apoptotic” role in neural precursor development and the differentiation of sensory organ precursor cells (Kanuka et al., 2005; Koto et al., 2009; Kuranaga et al., 2006). Several hypotheses have been suggested to explain why Dronc can selectively cleave development-related substrates but not induce apoptosis, including caspase compartmentalization and confined proteolytic activity. In support of the latter model, our observations support the possibility that neural precursor cells may restrain the proteolytic activity of Dronc by modulating of NADPH levels, allowing only a limited population of Dronc to bind to Dark and thereby become active. In further
investigations, it would be interesting to examine how the phosphorylation of Dronc S130 fluctuates during neural cell development.

4.3 FASN is involved in NADPH-mediated suppression of initiator caspases

In Chapter 3, we presented data from both Drosophila S2 cells and human ovarian cancer cells to argue that FASN plays an essential role in the metabolic control of apoptosis. In S2 cells, impeding the flux through FASN with a chemical inhibitor, orlistat, abrogates NADPH’s protective effects against CHX-induced apoptosis. Similarly, orlistat treatment lifted the NADPH-mediated inhibitory phosphorylation of caspase-2 (S164), resulting in caspase-2 activation and the subsequent ES-2 cell death.

Upon NADPH deprivation, flux through FASN will be presumably halted, leading to both a decrease in fatty acid production and an accumulation of precursors (e.g., acetyl-CoA and malonyl-CoA); however the effect responsible for directly causing death with NADPH deficiency remains unknown. Theoretically, we can treat cells with these metabolites, including acetyl-CoA, malonyl-CoA and different fatty acids, and see how they modulate the activation of initiator caspases. However, this type of study is limited by the fact that acetyl-CoA and malonyl-CoA are not cell permeable, nor have we identified (if there is/are any) the relevant fatty acid(s) produced from FASN.

Contradictorily, supplementation of several major products from FASN into cell culture medium, such as palmitic acid (C16:0) and stearic acid (C18:0), have been shown to induce apoptosis (Artwohl et al., 2004; Ji et al., 2005; Notarnicola et al., 2011), rather
than suppress cell death as our model predicts. Nevertheless, the presence of excess fatty acids inhibits FASN activity, shutting down the production of all kinds of fatty acid species, thereby resulting in fatty acid “deficiency” (Notarnicola et al., 2011). Therefore, we still cannot rule out the possibility that NADPH deprivation causes a deficit of certain FASN products, thereby inducing caspase activation and cell death. Further investigations utilizing metabonomics should allow us to assess global changes in different metabolite levels upon NADPH deprivation. Alternatively, a whole genome screen with the Dronc BiFC system we developed may help to elucidate the role of FASN in NADPH-mediated suppression of initiator caspases.

4.4 Mechanisms of cancer cell death induced by FASN inhibition

Our data presented in Chapter 3 also suggest that caspase-2 is required for the cell death observed with FASN inhibition in human ovarian cancer ES-2 cells. Several FASN inhibitors, including orlistat, cerulenin and C75, have all been shown to reduce tumor progression and survival in numerous cancer models, suggesting an oncogenic function of FASN (Flavin et al., 2011). In fact, forced expression of FASN directly transforms immortalized prostate epithelial cells into highly invasive tumors (Migita et al., 2009). Consistent with data from animal models, FASN was also proven to be highly expressed in cancers of various origins in clinic, including ovarian, prostate, gastric tract and breast cancers (Bandyopadhyay et al., 2006; Dowling et al., 2009; Flavin et al., 2011; Uddin et al., 2011; Zecchin et al., 2011; Zhou et al., 2003). Together, these findings
support the notion that FASN could be a drug target with great anti-cancer potential. However, the mechanism linking FASN inhibition and cell death remains elusive.

Several cellular alterations resulting from compromised FASN activity have been suggested as an origin of death signals, including DNA replication arrest, phospholipid deficiency, and ceramide production. Regardless of its origins, most cell death triggered by FASN inhibitors appears to be apoptotic in nature (Bandyopadhyay et al., 2006; Pizer et al., 1998; Zhou et al., 2003). Indeed, caspase-3, -9 and -8 have all been shown to be involved in cell death resulting from FASN inhibition in different contexts. However, it is surprising to find that caspase-2’s involvement has not been examined.

As presented in Chapter 3, we clearly observed caspase-2 and Bid cleavage upon FASN inhibition in several human ovarian cancer cell lines, including ES-2, 432 and 420. Although it has been suggested that Bid is cleaved by caspase-8 during apoptosis, downregulation of caspase-2 by siRNA significantly restored full-length Bid levels after the addition of the FASN inhibitor, orlistat. Most importantly, orlistat-induced cell death was greatly alleviated in caspase-2 deficient cells, confirming the necessity of caspase-2 in cell death mediated by FASN inhibition. Since FASN is overexpressed in most ovarian cancer cells, and its activity is required to maintain an NADPH-mediated inhibitory phosphorylation on human caspase-2 (at S164), it is possible that ovarian cancer cells escape from apoptosis, at least in part, by silencing caspase-2 through the metabolic regulation we observed.


4.5 Concluding remarks

Metabolism and apoptosis are clearly intertwined in vertebrates. In this dissertation, we demonstrate an unprecedented link between these two important biological processes in Drosophila. The mode by which NADPH metabolism impinges upon the fly apoptotic machinery also presents the first characterized regulation that acts in a DIAP1-independent manner. In the context of Drosophila development, our findings intriguingly suggest that NADPH metabolism may restrain the proteolytic activity of Dronc, permitting it to perform non-apoptotic role during neural precursor development. Thus, perturbation of this metabolic suppression directly results in neuronal developmental defects. In an attempt to elucidate the signaling pathway further, we found that FASN plays an evolutionarily conserved role in the NADPH-mediated suppression of initiator caspases. This observation also manifests the necessity of caspase-2 in ovarian cancer cell death resulting from compromised FASN activity, shedding light on the pharmacological mechanism of FASN inhibitors. Furthermore, the regulatory similarity between Dronc and caspase-2 was evident from these observations that caspase-2 appears to be necessary for cell death resulting from compromised FASN activity in ovarian cancer cells. Overall, these findings open the way for genetic studies to fully elucidate the pathways linking metabolism and caspase activation. The striking parallels between Dronc and caspase 2 lead us to postulate that intermediaries identified through dsRNA screening or genetics in Drosophila will provide strong clues to similar intermediaries in vertebrate cells. Therefore, the BiFC-based indication system we
developed will permit us to perform a genome-wide RNAi screening in the future, potentially identifying more missing pieces in the tangled regulatory puzzle that coordinates cell metabolism and programmed cell death.
References


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