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IAP antagonist Grim mediates cell death through divergent pathways

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IAP antagonist Grim mediates cell death through divergent pathways

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Apoptosis is an evolutionarily conserved process, carried out through proteolytic cascades that lead to activation of cysteinyl aspartate-specific proteases (caspases), which in turn cause cell self-destruction. While caspases are activated upon various death stimuli or developmental cues, inhibitor of apoptosis (IAPs) can prevent caspase-mediated cell death, not only through direct binding and inhibition, but also by directly ubiquitinating caspases, which inactivates them or targets them for degradation. In flies and mammals, cell death is facilitated or enhanced by the accumulation of "IAP antagonists", which promote cell death by utilizing their N-terminal IAP binding motifs (IBMs) to interact with IAPs, to displace caspases from IAP-binding, and to promote IAPs autoubiquitination. Herein, we demonstrate that a *Drosophila* IAP antagonist, Grim, induces cell death through divergent mechanisms: an IBM-dependent pathway, requiring a crosstalk with *Drosophila* IAP1 (DIAP1), and an IBM-independent manner,

involving Grim's C-terminus for translational repression. We discovered that Grim is a target for caspase-mediated cleavage at Grim's C-terminus (Asp132). Grim loses its only lysine (Lys 136) after cleavage, but acquires a longer half-life due to inability of DIAP1 to ubiquitinate it, which in turn leads to greater cell death. This novel regulation allows increased levels of Grim to efficiently kill through an IBM-dependent mechanism that derepresses caspase inhibition. However, in addition to IBM-dependent cell death, Grim is able to induce caspase activation independent of its IBM. In our studies, we have demonstrated that IBM-independent cell death requires Grim's C-terminal domain as well as the characteristic Grim Helix 3 (GH3) domain. Importantly, Grim's C-terminus localizes Grim to distinct cytoplasmic granules, i.e. P-bodies, where translation is repressed due to the accumulation of protein components that regulate mRNA translation or stability. Knockdown of a P-body-localized translational repressor, Me31B, largely reduced IBM-independent cell death. Taken together, IBM-independent cell death occurs, at least in part, through Me31B-mediated translational repression, requiring the localization of Grim to P-bodies via Grim's C-terminus. Overall, we have discovered that the interplay between caspases and the proteasome system creates an intricate regulatory system for maintaining Grim at low levels in the cell under normal conditions. Once needed, Grim levels accumulate rapidly and induce cell death efficiently through IBM-dependent destruction of DIAP1 and derepression of caspases as well as through IBM-independent translational repression.

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List of Abbreviations

Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
Ala	Alanine
Apaf-1	Apoptotic protease-activating factor-1
Asp	Aspartic acid
BIR	Baculovirus inverted repeat
Caspase	Cysteinyl aspartate-specific protease
CHIP	Conjugated C-terminal Hsc70-interacting protein
CHX	Cycloheximide
c-IAP1/2	Cellular inhibitor of apoptosis 1/2
DAPI	4',6-diamidino-2-phenylindole
DARK	*Drosophila* Apaf-1 related killer
Dcp-1	Death caspase-1
DCP1	Decapping protein 1
DIAP1	*Drosophila* inhibitor of apoptosis 1
DIC	Differential interference contrast
DIBALO	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DrICE	*Drosophila* Interleukin-1β-converting enzyme
DRONC	*Drosophila* Nedd-2 like caspase
dsRNA	double-stranded RNA
EDC	Enhancer of mRNA decapping
eIF4E	Eukaryotic initiation factor 4E

FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GH3	Grim helix-3
GST	Glutathione-S-transferase
Hid	Head involution defective
HSC70	Constitutive heat shock protein 70
Htra2	High temperature requirement A2
IBM	IAP-binding motif
Lys	Lysine
Me31B	Maternal expression at 31B
Met	Methionine
MOMP	Mitochondrial outer membrane permeabilization
NF-κB	Nuclear factor -κB
Ni-NTA	Nickel-nitrilotriacetic acid
PARP	Poly(ADP-ribose) polymerase
P-BODY	Processing body
PBS	Phosphate buffered saline
RING	Really interesting new gene
Smac	Second mitochondria-derived activator of caspase
UB	Ubiquitin
UTR	Untranslated region
UV	Ultraviolet
WT	Wild-type
XIAP	X-linked inhibitor of apoptosis
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone

CHAPTER 1

Introduction

1.1 Programmed cell death

Apoptosis, or programmed cell death, is a highly regulated process involving proteolytic cascades that lead to cell self-destruction characterized with well-defined morphology, such as DNA fragmentation, chromatin condensation, cell membrane blebbing, cytoskeletal collapse, and the formation of apoptotic bodies (Kerr et al., 1972). This evolutionarily conserved process is required during the development and morphogenesis of metazoans, from *C. elegans* to *Drosophila* to mammals. In addition to apoptosis, there are other forms of cell death, including necrosis, autophagic death, and necroptosis. Necrosis is unprogrammed and uncontrolled cell lysis, which can cause the massive release of cytokines and a strong proinflammatory response. Necroptosis has implications in ischemia reperfusion injury and systemic inflammatory response syndrome and involves the activation of unique signaling cascades in cells that result in necrosis, although the detailed mechanisms remain unclear (Kaczmarek et al., 2013; Linkermann and Green, 2014). Autophagy can act as a tumor suppressor or cause chemoresistance depending the stage of cancer development and is still under investigation (Guo et al., 2013; White, 2012).

Conversely, apoptosis has been widely studied because of its implications in many human diseases, such as cancer, neuron degeneration, autoimmune disorders, and viral infection. Several biomarkers have been utilized to characterize apoptotic cell death, including for example, caspase activation, redistribution of phosphoytidylserine (PS)

from inner-to-outer plasma membrane, changes in mitochondrial membrane permeability, and caspase-dependent activation of DNases (Enari et al., 1998; Fadok et al., 1992; Kroemer and Reed, 2000; Naito et al., 1997). Dismantling of structural and functional proteins during apoptosis is usually carried out by cysteine proteases, known as caspases. Cells can fight for their survival through expression of inhibitor of apoptosis (IAP) proteins, which diminish caspase activity inside the cell and inhibit apoptosis. However, when cells are under stress, are treated with chemotherapeutic agents, or are destined to die during development, IAP antagonists can be released from specific organelles or can be upregulated to inhibit the function of IAPs (Fig. 1.1). Alterations in the balance among caspases, IAPs, and IAP antagonists can effectively enhance or reduce cell death, and thus, are often viewed as targets for drug design. Structures are available for many caspases, IAPs, and IAP antagonists, but the detailed mechanisms through which they are regulated are not fully understood. This study will investigate the function of IAP antagonists and how they regulate cell death in both traditional and unconventional ways. Understanding the regulatory pathways of apoptosis may reveal more targets for future drug design in order to overcome human diseases, such as cancer or neurodegeneration.

1.1.1 The apoptotic pathway in mammals

Two well-defined apoptotic signaling cascades are present in mammals: the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways. The two pathways are activated by different cellular insults, and ultimately death signals are amplified and executed through the activation of caspases. Developmental cues, UV irradiation, DNA damaging agents, and reactive oxygen species can activate the intrinsic pathway through perturbation of mitochondria. Loss of mitochondrial outer membrane potential and

release of cytochrome *c* from the intermembrane space into the cytoplasm leads to the formation of a large protein complex, known as the apoptosome. Apoptotic protease-activating factor-1 (Apf-1) undergoes oligomerization, in a cytochrome *c* and ATP/dATP-dependent manner, into a heptameric complex that recruits and activates caspase-9 (Acehan et al., 2002; Cain et al., 1999). Caspase-9 acquires proteolytic activity once bound to the apoptosome complex and can then directly activate the downstream effector caspases-3 and -7, which are responsible for the execution of apoptosis (Malladi et al., 2009). In addition to cytochrome *c*, Smac/DIABLO (Second mitochondria-derived activator of caspase/direct IAP binding protein with low pI) and Omi/HtrA2 (High temperature requirement A2) are also released from mitochondria and potentiate cell death by antagonizing the IAPs (Fig. 1.1) (Du et al., 2000; Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2000; Wu et al., 2000). The extrinsic pathway, on the other hand, utilizes death ligands to activate death receptors resulting in the formation of a multimeric death inducing signaling complex (DISC) that recruits and activates caspase-8, which in turn activates the downstream effector caspases-3 and -7. In some cells, caspase-8-dependent cleavage of the BH3 only protein Bid helps induce cell death but also engages the intrinsic pathway.

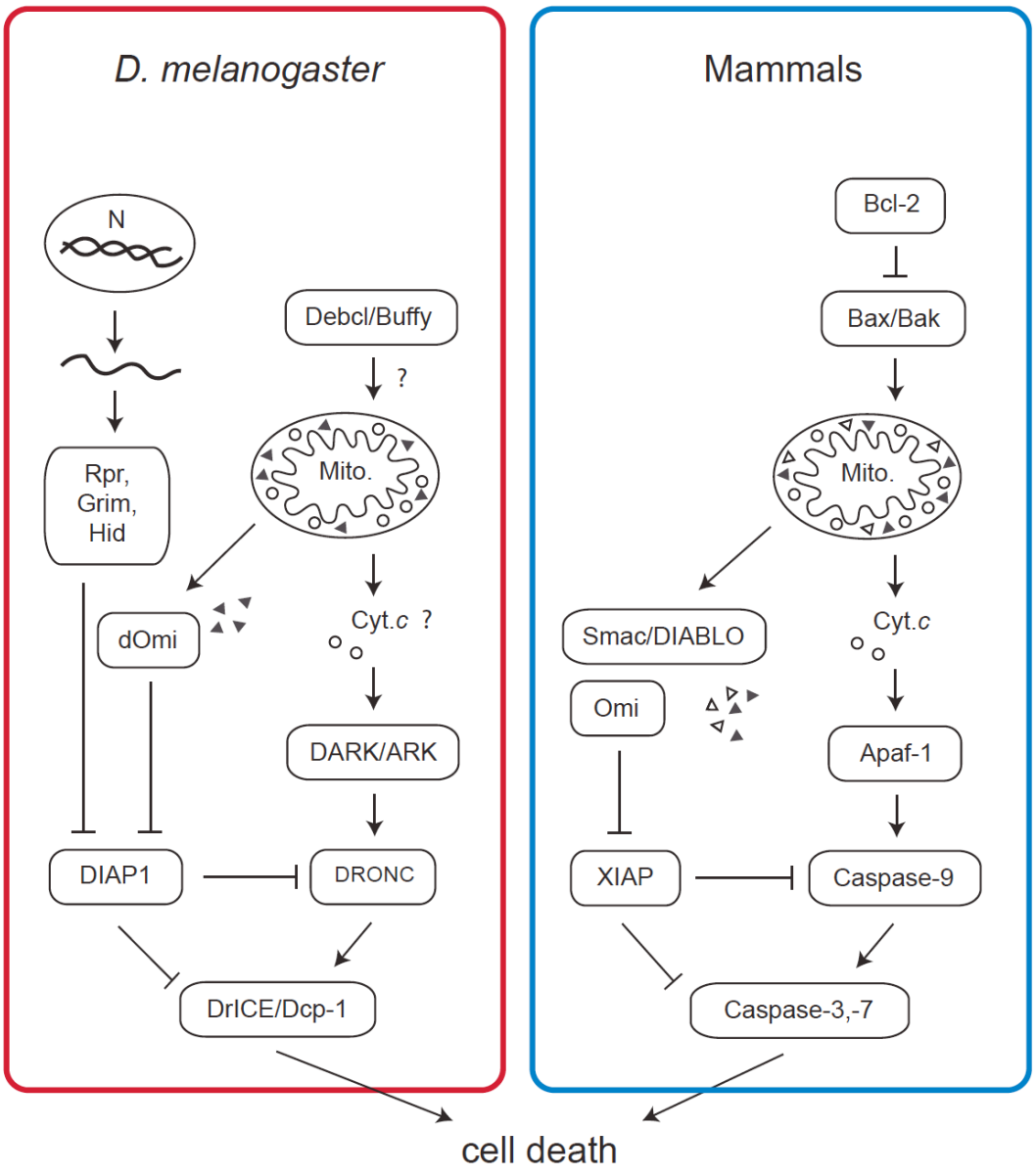

Figure 1.1 The conserved intrinsic apoptosis pathway. Many of the core proteins involved in regulating cell death share functional or structural similarities in *Drosophila* and mammals. In mammals, pro-apoptotic Bax and/or Bak permeabilize mitochondria, which result in the release of cytochrome *c* (cyt. *c*) and mitochondrial IAP antagonists, Smac/DIABLO and Omi. Cyt. *c* triggers the assembly of the apoptosome complex, which activates caspase-9 and its downstream effectors, caspase-3 and caspase-7. Even though active caspases can be inhibited by XIAP, IAP antagonists compromise XIAP, which leads to caspase reactivation and apoptosis. In the case of *D. melanogaster*, once mitochondria are damaged, dOmi is released to antagonize DIAP1 function so that active DRONC, DrICE, Dcp-1 can execute cell death. Other than dOmi, IAP antagonists, such as Reaper, Grim, and Hid are also upregulated upon stress. (Modified from Parrish *et al.*, 2013)

1.1.2 The apoptotic pathway in flies

The intrinsic pathway shares strong similarity between flies and mammals, but it also appears to have significant differences (Fig. 1.1). *Drosophila* contains BCL2 family members, but the role of mitochondrial damage in cell death remains highly controversial (Abdelwahid et al., 2007; Arama et al., 2003; Arama et al., 2006; Challa et al., 2007; Dorstyn et al., 2004; Galindo et al., 2009; Means et al., 2006; Varkey et al., 1999). *Drosophila* Nedd-2-like caspase (DRONC), the mammalian caspase-9 homologue, requires *Drosophila* Apaf-1-related killer (DARK) for its activation, even though cytochrome *c* is not released from mitochondria or required to form the apoptosome complex (Dorstyn et al., 2004; Dorstyn et al., 2002; Means et al., 2006; Yuan et al., 2011; Zimmermann et al., 2002). Nevertheless, once DRONC acquires catalytic activity, it cleaves and activates effector caspases, *Drosophila* Interleukin-1β-converting enzyme (DrICE) and death caspase-1 (Dcp-1) (Fig. 1.1).

On the other hand, the *Drosophila* Omi homologue, dOmi, can be released from mitochondria to antagonize *Drosophila* IAP1 (DIAP1) as well as to use its serine protease activity to cleave it (Challa et al., 2007). Besides the mitochondria-localized dOmi, other IAP antagonists (e.g., Reaper, Grim, and Hid) in *Drosophila* are upregulated when they are needed during embryo development or when cells are damaged (Chen et al., 1996; White et al., 1994) (Fig. 1.1). They inhibit IAPs and therefore facilitate caspase-mediated cell death. Despite some of the differences between mammals and flies, the strong functional and structural similarities in their apoptotic pathways, as well as the strength of genetic screening in *Drosophila* make it a good model system to study cell death. What is needed are more biochemical studies in flies to carefully dissect the

differences between flies and mammals, which may provide new insights into how cell death is executed. Apparent differences in flies may well point to previously unidentified but important nuances to existing pathways in humans.

1.2 Regulators in apoptotic pathways

1.2.1 Caspases

The first member identified in the cysteinyl aspartate-specific protease (caspase) family was the human interleukin-1β-converting enzyme (ICE), which plays a role in inflammation. Based on sequence and functional homology, 11 caspases were identified in the human genome and designated as caspases-1 to -10 and caspase-14. They can be grouped into pro-inflammatory (caspases-1, -4, -5, -14) and pro-apoptotic caspases (caspases-2, -3, -6, -7, -8, -9, -10), but it is worth noting that some of these caspases are also involved in differentiation or migration (Fernando et al., 2002; Helfer et al., 2006; Li and Yuan, 2008; Miura et al., 2004). In terms of structure and domain organization, proapoptotic caspases are divided into initiator caspases (caspases-2, -8, -9, -10) and effector caspases (caspases-3, -6, -7) (Fig. 1.2). All caspases consist of a prodomain (3-23 kDa), followed by a large subunit (20 kDa) and a small subunit (10 kDa) (Fig. 1.2). Initiators possess long prodomains with the ability to interact with adaptor proteins to form large multimeric complexes and therefore acquire catalytic activities. Active effector caspases are heterotetramers. Two single chain zymogens are processed into two large and two small subunits, with the large subunits bearing the catalytic cysteines. Cleavage of the intersubunit linker between the large and small subunits is usually carried out by initiator caspases, resulting in conformational changes that form the substrate-

binding pocket (Chai et al., 2001; Pop and Salvesen, 2009). Most caspases recognize tetrapeptide sequences in substrates, classified as P4-P3-P2-P1 upstream of the scissile bond (Pop and Salvesen, 2009). Caspases cleave after the P1 Aspartate, and DXXD sequences are usually preferred by effector caspases-3/-7. In the P2 position, caspases-3/-7 prefer small hydrophobic residues (Ala, Val), while in the P3 position, favor the formation of hydrogen bonds (Glu). A small uncharged amino acid is usually preferred for the P1' position (the first amino acid after the cleavage site). The DEVD sequence present in poly(ADP-ribose) polymerase (PARP) serves as a great example of a caspase-3/-7 substrate (Lazebnik et al., 1994). As a result, fluorescence substrates DEVD-AMC or DEVD-AFC are often used to measure the enzymatic activity of caspases-3 and -7 *in vitro* (Stennicke et al., 2000; Tewari et al., 1995).

Drosophila caspases are also very similar to mammalian caspases structurally and functionally. DRONC acts upstream of DRICE and Dcp-1 (Fig. 1.1) and is activated through a DARK-dependent manner, but it also has a different regulation from caspase-9. The mechanism of DRONC activation is still controversial. DRONC is activated by the DARK apoptosome complex through induced dimerization (Snipas et al., 2008) or autoprocessing between the large and small subunit (Muro et al., 2004). During apoptosis, cytochrome *c* did not release from mitochondria and addition of cytochrome *c* to fly cell lysate failed to activate DRONC, which indicates cytochrome c is not required for the assembly of fly apoptosome complex (Dorstyn et al., 2004; Dorstyn et al., 2002; Means et al., 2006; Zimmermann et al., 2002). However, *in vitro* reconstituted fly apoptosome failed to activate DRONC unless cell extracts were added, indicating that additional molecules are required for DARK-mediated DRONC activation (Dorstyn and

Kumar, 2008). DRONC is a unique caspase that prefers to cleave after either a glutamic acid or an aspartic acid, whereas most caspases catalyze cleavage only after an aspartic acid (Hawkins et al., 2000; Snipas et al., 2008). It has been suggested that DRONC exists in the cell as a constitutively active form and is constantly bound to and ubiquitinated by DIAP1 (Muro et al., 2002). Hormone ecdysone induces metamorphosis and upregulation of DRONC transcripts to remove obsolete tissues (Waldhuber et al., 2005). Homozygous *dronc* mutants exhibit a growth delay during the larval stage and die at early pupariation (Waldhuber et al., 2005).

Drosophila also has a caspase-8 homologue, Dredd, but its role appears to be more important in regulating innate immunity, especially through the NF-κB pathway. DIAP2-dependent ubiquitination of Dredd allows Dredd to be fully activated, which finally leads to Relish/NF-κB cleavage and activation (Meinander et al., 2012). The functions of the remaining fly caspases (Damm, Strica, and Decay) are less clear, but Strica and Decay appear to play an important role in Hid-mediated death (Leulier et al., 2006).

Two main effectors, DrICE and Dcp-1 are very similar to caspases-3 and -7 (Fig. 1.2), and their activities can also be measured using the substrate DEVD-AMC. They are both cleaved and activated by DRONC and can be inhibited by the viral anti-apoptotic protein p35 (Lannan et al., 2007; Snipas et al., 2008). DRICE and Dcp-1 share functional redundancy during fly embryo development, but they also have specific roles (Muro et al., 2006). Animals lacking *drice* show less cell death in the embryonic nervous system but still survive at the larval stage; the majority (80%) of null mutants, however, die before the end of the pupal stage (Muro et al., 2006). The viable adults are fertile, even though

there is a partial failure to undergo sperm individualization, indicating that DrICE participates in this non-apoptotic process (Muro et al., 2006). Although *dcp-1* mutant flies are viable and fertile (Laundrie et al., 2003), the loss of *drice* and *dcp-1* is lethal (Muro et al., 2006).

Effectors are responsible for cleaving the majority of cellular protein substrates, including structural proteins, adhesion molecules, cell cycle regulators, transcription factors, signal transduction players, and cell death modulators (Luthi and Martin, 2007). After cleavage, some proteins are inactivated, some exhibit an altered function due to the loss of a regulatory or binding domain, and some are rendered more active due to the removal of an inhibitory domain (Cheng et al., 1997; Emoto et al., 1995; Plesca et al., 2008; Semple et al., 2007). Many of these proteins also become susceptible to complete degradation, as in the case of the caspase inhibitor and E3 ubiquitin ligase, *Drosophila* inhibitor of apoptosis 1 (DIAP1), which is degraded via the N-end rule following removal of its N-terminus by caspases (Ditzel et al., 2003).

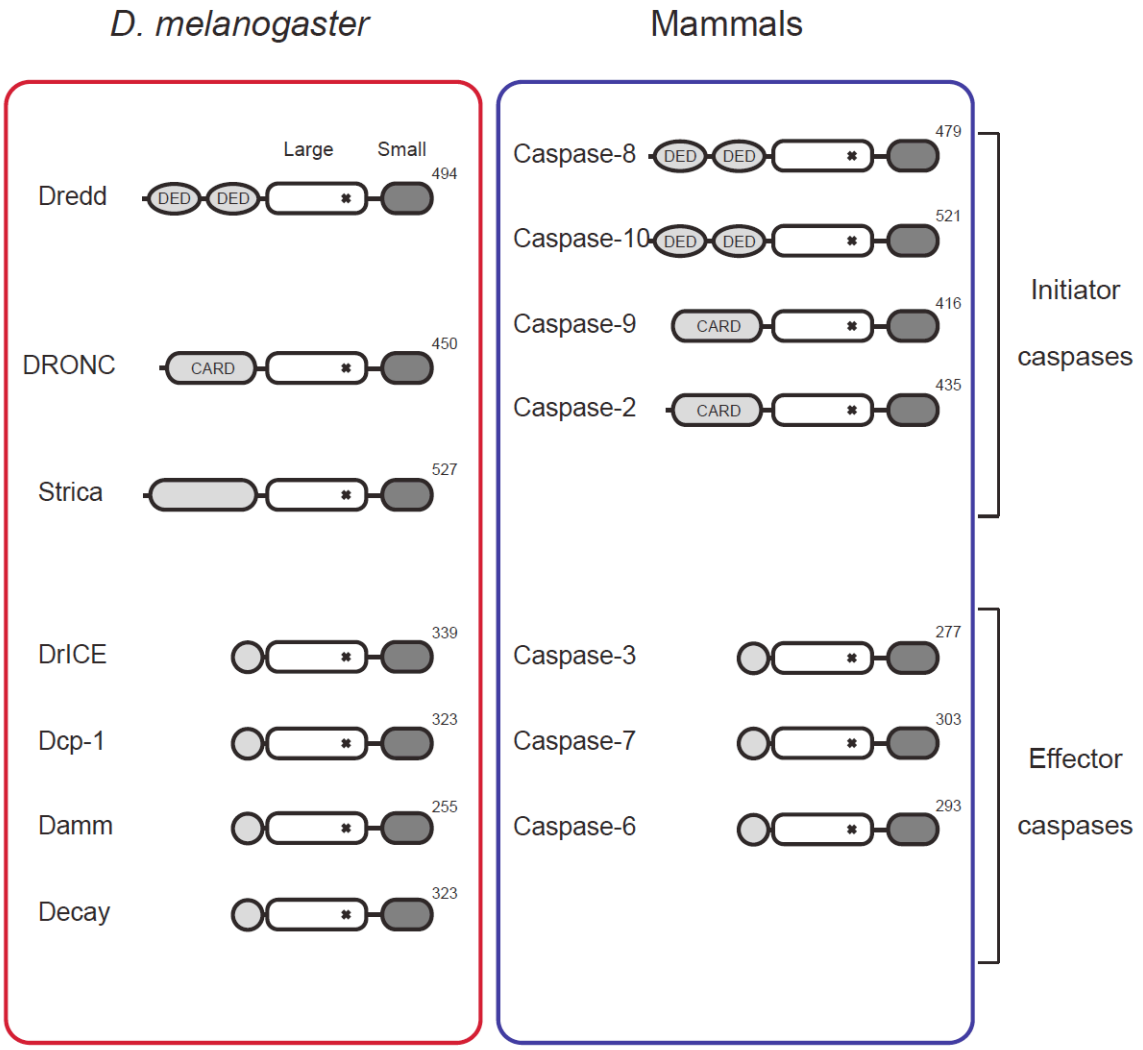

Figure 1.2 Initiator and effector caspases in *Drosophila* and mammals. Caspases are composed of a prodomain (represented as light gray), followed by a large (white color) and a small (dark gray) subunit with the active cysteine (represented as an asterisk) located near the C-terminus of the large subunit (not drawn to scale). Initiator caspases have a longer prodomain to interact with adaptor proteins, while the prodomains in the effectors are relatively short and their functions are largely unknown. DrICE and Dcp-1 are caspase-3 or caspase-7-like proteins in *Drosophila*, while DRONC is a caspase-2 or caspase-9-like protein in *Drosophila*. (Modified from Riedl and Shi 2004)

1.2.2 Inhibitor of apoptosis (IAP) proteins

The first IAP was cloned two decades ago from virus-infected insect cells (Crook et al., 1993). It was found to suppress apoptosis when expressed in cells due to the presence of characteristic zinc-finger like motifs, named Baculoviral IAP repeat (BIR) domains, which mediates protein-protein interactions (Crook et al., 1993). IAPs were later found not only in viruses, but also in flies, and mammals (Salvesen and Duckett, 2002) (Fig. 1.3). One IAP molecule can contain multiple BIR domains through which it interacts with specific proteins. Many IAPs, however, also possess a Really Interesting New Gene (RING) domain (Fig. 1.3), which allows them to post-translationally modify themselves or their binding partners through ubiquitination or neddylation (Broemer et al., 2010; Zhuang et al., 2013).

The BIR domain contains ~70 amino acids with a consensus sequence of $CX_2C\ldots W\ldots_{16}HX_6C$ (C, cysteine; W, tryptophan; H, histidine, and X, any amino acid). It forms an unusual hydrophobic pocket that often only allows a conserved N-terminal sequence in its substrates, known as the IAP binding motif (IBM), to bind into the pocket. An internal IBM in caspase-9 is rendered following cleavage between its large subunit and small subunit. Exposure of the IBM with an amino terminus of the small subunit targets caspase-9 for inhibition by X-linked IAP (XIAP) (Srinivasula et al., 2001). XIAP interacts with active caspases-3 and -7 through its BIR2 domain and an adjacent linker region (Huang et al., 2001; Riedl et al., 2001; Scott et al., 2005). For our studies, we have mainly focused on *Drosophila* IAP1 (DIAP1), as it has important roles in preventing cell death and, in many aspects, acts like XIAP. DIAP1 similarly interacts with active DrICE through the BIR1 domain and with DRONC through its BIR2 domain.

IAP antagonists are another IBM-containing family of proteins that potentiate cell death by interacting with BIR domains and antagonizing inhibition of caspases by IAPs (Fig 1.4*A*). Structural analyses reveal that mutation of key tryptophan or histidine residues in the BIR domains of IAPs disrupt their ability to interact with their substrates. We utilized mutagenesis in our studies to investigate the functions of different BIR domains in DIAP1. Conversely, simply blocking the N-terminal alanine in the IBM of IAP antagonists, or replacing it with a glycine, can destroy the IBM (Wright and Clem, 2002). We have used this approach to study the IBM-independent functions of Grim.

Drosophila IAP2 (DIAP2) and mammalian cellular IAP1 (c-IAP1) and c-IAP2 can participate in regulating cell death, but are more important for modulating immune signaling (Gyrd-Hansen and Meier, 2010). An *in vitro* study showed that c-IAP1 and c-IAP2 interact with caspases-3 and -7, but do not inhibit caspase activity (Eckelman and Salvesen, 2006). In fact, c-IAPs protect cells from apoptosis probably through a mechanism depending upon their E3 ligase activities (Choi et al., 2009; Samuel et al., 2006). c-IAP1 can ubiquitinate caspases-3 and -7, which may promote caspase degradation and facilitate cell death (Choi et al., 2009). Indeed, while DIAP2 is dispensable for fly development, it is required for NF-κB signaling and in defending against Gram-negative bacteria (Huh et al., 2007; Paquette et al., 2010). Although downregulation of DIAP2 sensitizes cells to apoptosis, downregulation of DIAP1 directly activates caspases and induces apoptosis (Zimmermann et al., 2002).

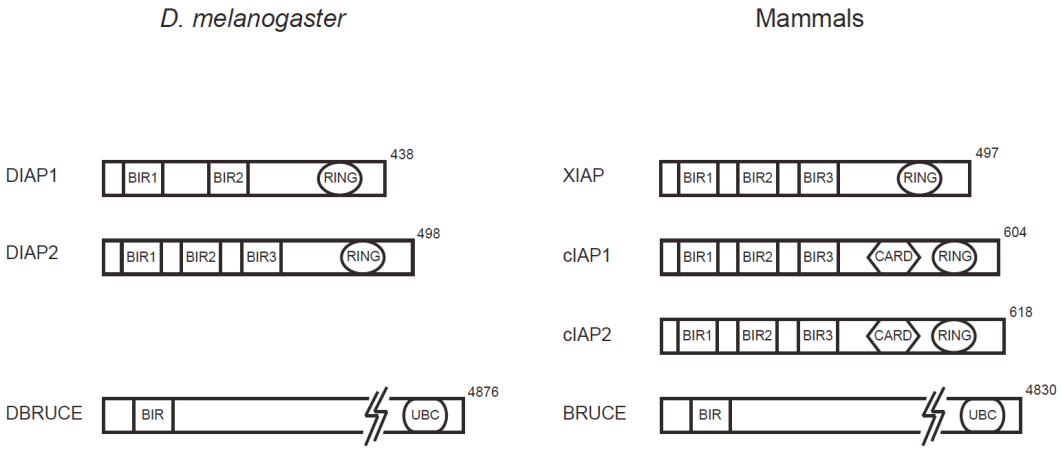

Figure 1.3 Comparison of *Drosophila* and mammalian IAPs. There are at least eight IAPs in mammals, and four in flies, but only functional homologues are listed in this figure (not drawn to scale). All IAPs have at least one BIR domain, and some have RING or UBC domains that are related to post-translational modifications. DIAP1 is the functional homologue of XIAP, and its BIR1 domain is equivalent to the BIR2 of XIAP, while its BIR2 domain is functionally similar to the BIR3 of XIAP. DIAP2 plays important roles in innate immunity and is more similar to c-IAP1 and c-IAP2. BRUCE participates in cell death regulation requiring a functional UBC domain. (Modified from Srinivasula and Ashwell, 2008)

1.2.3 DIAP1

DIAP1 is encoded by *thread* (th) and is absolutely required for embryo development in *Drosophila*. Inhibition of cell death occurs through physical interaction with caspases and requires ubiquitination of DIAP1 binding substrates through its E3 ubiquitin ligase activity. Indeed, physical interaction alone is insufficient to account for all of DIAP1's function, as mutation of its RING domain alters its anti-apoptotic activity (Lisi et al., 2000; Yokokura et al., 2004). Loss of DIAP1 in fly eyes causes rapid cell death because DIAP1 normally inhibits a constantly processed active form of DRONC by binding to its prodomain, ubiquitinating it, and keeping its levels low in healthy cells (Meier et al., 2000; Muro et al., 2002). Even though DRONC lacks a classic IBM sequence, its prodomain contains a specific sequence that is nevertheless able to interact with the BIR2 domain in DIAP1 (Chai et al., 2003). It is unclear what type of ubiquitin chains are conjugated to DRONC, but ubiquitinated DRONC is not degraded, implying that DIAP1 may catalyze a K63-based ubiquitination (Lee et al., 2011). In contrast, DIAP1 does not interact with proDrICE. DIAP1 inhibits active DrICE through its BIR1 domain, and reportedly reduces DrICE activity through nondegradative polyubiquitination, although DIAP-BIR1 has been shown to inhibit DRICE independent of ubiquitination (Challa et al., 2007; Ditzel et al., 2008; Yan et al., 2004). The N-terminal 10 amino acid fragments in Reaper, Grim, or Hid have higher affinity toward the BIR domains than DrICE or DRONC, so that DrICE and DRONC are displaced from the BIR binding pocket *in vitro* (Chai et al., 2003; Yan et al., 2004). In fact, these three proteins show differential binding toward two BIR domains when the full-length protein was used. Reaper and Grim interact with both BIR1 and BIR2, whereas Hid

preferentially binds to the BIR2 domain (Zachariou et al., 2003). *In vivo* studies also confirmed that Reaper, Grim, or Hid were capable of interacting with DIAP1 and efficiently promote cell death (Goyal et al., 2000; Lisi et al., 2000).

As already noted, Reaper, Grim, or Hid promotes cell death through mechanisms unrelated to the displacement of DrICE or DRONC from BIR domains. The three proteins also directly regulate DIAP1 levels by stimulating RING-dependent DIAP1 autoubiquitination (Ryoo et al., 2002; Yoo, 2005; Yoo et al., 2002). Autoubiquitination of DIAP1 requires two ubiquitin-conjugating enzymes (E2), Morgue and UbcD1 (Hays et al., 2002; Ryoo et al., 2002; Wing et al., 2002). DIAP1 stability is also regulated through other post-translational modifications. *Drosophila* IKK-related kinase (DmIKKε) and Hippo kinase phosphorylate DIAP1 and promote DIAP1 degradation, but the phosphorylation sites and detailed mechanism remains unknown (Harvey et al., 2003; Kuranaga et al., 2006).

Other than caspases and DIAP itself, DIAP1 also promotes the ubiquitination of Reaper, Grim, and Hid (Olson et al., 2003b; Yeh and Bratton, 2013), but the fate of these ubiquitinated proteins is not always clear. Whether Grim is ubiquitinated through a non-lysine residue like Reaper is unknown (Domingues and Ryoo, 2012). Besides, the E2 ubiquitin-conjugating enzyme that facilitates ubiquitination is also unclear. In our studies, we will focus on DIAP1-dependent Grim ubiquitination.

Table 1.1 Differences in Reaper, Grim, and Hid

	Reaper	Grim	Hid
IBM	+	+	+
Interaction with DIAP1	BIR1 and BIR2 domains	BIR1 and BIR2 domains	BIR2 domain
Cell death prevented by DIAP1 overexpression	+	-	+
Cell death prevented by DIAP2 overexpression	+	-	+
Cell death prevented by zVAD-fmk	+	+	+
GH3 domain	+	+	-
IBM-independent cell death	+	+	Not reported
Inducer-Wingless	+	+	+
Inducer-UV, irradiation	+	Not tested	Not tested
Inducer-ecdysone	+	Not tested	+

1.2.4 IAP antagonists

The first IAP antagonists were discovered through a genetic screen in *Drosophila*. Loss of about 300 kb of DNA on the third chromosome, the H99 locus, completely prevented embryonic apoptosis, resulting in the accumulation of excess cells in the central nervous system and a failure of embryos to hatch (White et al., 1994). However, H99 embryos were still capable of undergoing apoptosis even though death was not activated spontaneously during the development, which suggests that genes in the H99 locus are likely to be activators but not executors of apoptosis (White et al., 1994). Reaper was identified first in this screen, but *hid* and *grim* were later shown to be present in the same H99 locus and functions in similar ways as apoptosis inducers during *Drosophila* development. Each gene, *reaper*, *grim*, or *hid*, alone was capable of inducing apoptosis when expressed in cultured cells, but was insufficient to rescue loss of the entire H99 locus (Chen et al., 1996; Grether et al., 1995; White et al., 1994). It was suggested that the protein products encoded by the three genes share functional redundancy, especially during early embryo development but also have unique roles later in development during metamorphosis. Table 1 summarizes the main similarities and differences among the three proteins. Yet another IAP antagonist, *sickle*, is located near *reaper*, and antagonizes DIAP1 (Srinivasula et al., 2002).

A major feature of IAP antagonists is their ability to inhibit IAPs and potentiate cell death by utilizing their IBM domain. Fig. 1.4*A* lists the IBM sequences in mammals and *Drosophila*. The IBM in IAP antagonists usually has a higher affinity for the BIR domain than do the caspases, such that they can efficiently compete with caspases and release them from the IAPs (Chai et al., 2003; Yan et al., 2004). The first four amino

acids in the IBM are highly conserved, especially the first amino acid, alanine (Fig. 1.4*A*). For some IAP antagonists, such as Omi (which is conserved in *Drosophila*) and Smac/DIABLO, removal of the mitochondrial targeting sequence, following import into mitochondria, exposes their N-terminal IBM (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001). Following MOMP, the IAP antagonists are released into the cytoplasm where they interact with IAPs, displace active caspases and enhance the autoubiquitination of IAPs (Martins et al., 2002; Suzuki et al., 2001). Other IAP antagonists, like Reaper, Grim, and Hid, have an IBM at the N-terminus, immediately following the initiator methionine. This methionine is usually removed by aminopeptidase so that the IBM is exposed to perform its function (Cutforth and Gaul, 1999; Wright and Clem, 2002). These IAP antagonists are generally upregulated through transcriptional control after receiving key developmental cues or cellular stress.

Interestingly, the strict IBM region covers only four amino acids of the protein, raising questions as to the function of the remaining portion of the protein. Indeed, even though an IBM is an important feature for IAP antagonists, many of these proteins have other non-IBM-related functions that appear to be required for maximum killing. *Drosophila* Omi is a serine protease capable of degrading DIAP1 without its IBM (Challa et al., 2007). Reaper can induce cell death through an IBM-independent, but C-terminus-dependent manner with the several proposed mechanisms (Chen et al., 2004; Freel et al., 2008).

The C-terminus of Reaper comprises a characteristic amphipathic α-helix (Grim Helix 3, or GH3 domain), shared homology with Grim (Fig. 1.4*B*). The function of GH3 domain is under intensive investigation, but the detailed mechanisms remain

controversial. Domingues and Ryoo (2012) found that Reaper uses its GH3 and IBM to interact with the *Drosophila* IAP, dBruce, which leads to the turnover of Reaper through a non-lysine-dependent ubiquitination by dBruce. Reaper was reported to localize to mitochondria through the GH3 domain that allowed it to insert into the mitochondrial outer membrane through a protein-lipid interaction that did not require any other protein component (Olson et al., 2003b). The same group later found that Reaper interacts with a dMFN on the outer mitochondrial membrane and inhibits mitochondrial fusion (Thomenius et al., 2011).

However, Steller and colleagues showed that Reaper does not associate with mitochondria directly. Rather, it is the interaction between Hid and Reaper that brings Reaper to the mitochondrial outer membrane, as Hid possesses a mitochondrial targeting sequence (Sandu et al., 2010). They proposed that the real function of the GH3 domain in Reaper is to interact with proteins, such as Hid. Similarly, Chen and co-workers (2004) found that Reaper induces cell death through two distinct mechanisms: IBM-mediated caspase activation, and C-terminal fragment-dependent killing. Interestingly, the GH3 domain alone did not kill the cell nor colocalize with mitochondria (Chen et al., 2004). Even though the localization of Reaper remains controversial, it nevertheless ultimately induces mitochondrial damage and cell death without the IBM. It also associates with a ribosomal small subunit to inhibit protein translation, which eventually results in cell death. Interestingly, Bunyaviral nonstructural proteins (NSsSa and NSsLac) share sequence similarities with the GH3 region, but not the IBM, and they induce apoptosis and translational repression in a fashion that is very similar to the GH3 domain in Reaper (Colon-Ramos et al., 2003). It has been suggested that the bi-functional Repear protein

might have evolved into two separate proteins, but, if so, the mammalian GH3-like proteins have not yet been identified (Colon-Ramos et al., 2003). Thus, the IBM-independent activities of IAP antagonists are important but unclear. To understand their functions and regulation is therefore a goal of the laboratory, and the mammalian homologues of these proteins and their overall functions may be revealed through the study of IBM-less versions of Reaper, and Grim.

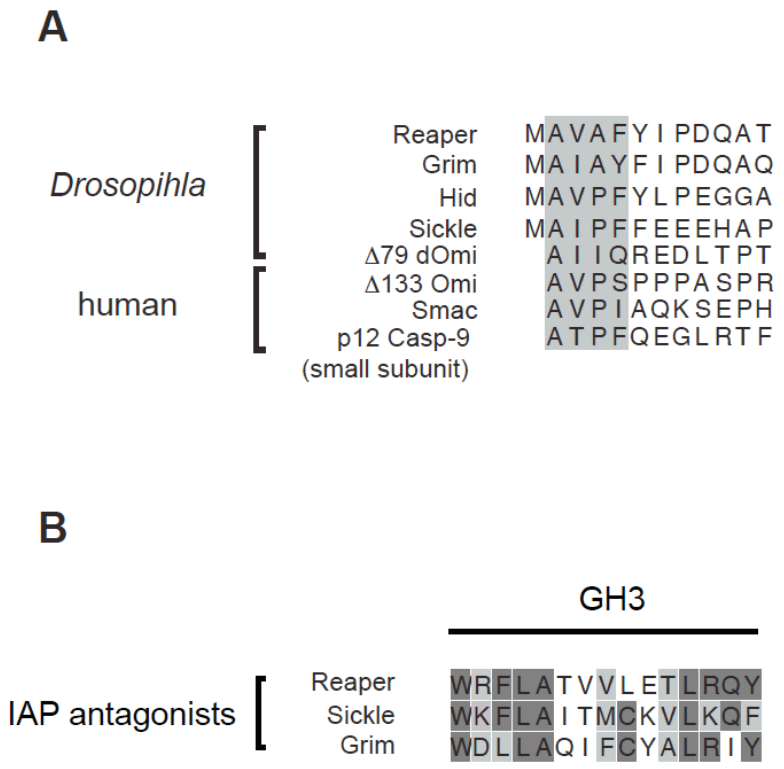

Figure 1.4 The IAP-binding motif (IBM) and the GH3 domain. (*A*) Flies have several IAP antagonists with N-terminal IBMs (highlighted in gray), the IBMs are exposed following removal of the initiator methionine by amino methylpeptidases. *Drosophila* Omi, human Omi, and Smac have internal IBMs that are exposed by the removal of mitochondrial targeting sequences. Human caspase-9 has the IBM on the N-terminus of the p12 small subunit that is exposed after a caspase-mediated cleavage event. (*B*) The GH3 domain shares sequence similarities among Grim, Reaper, and Sickle. Identical sequences are indicated in dark gray, while residues with some similarity are represented in light gray. (The lower panel is adapted from Claveria *et al*., 2002)

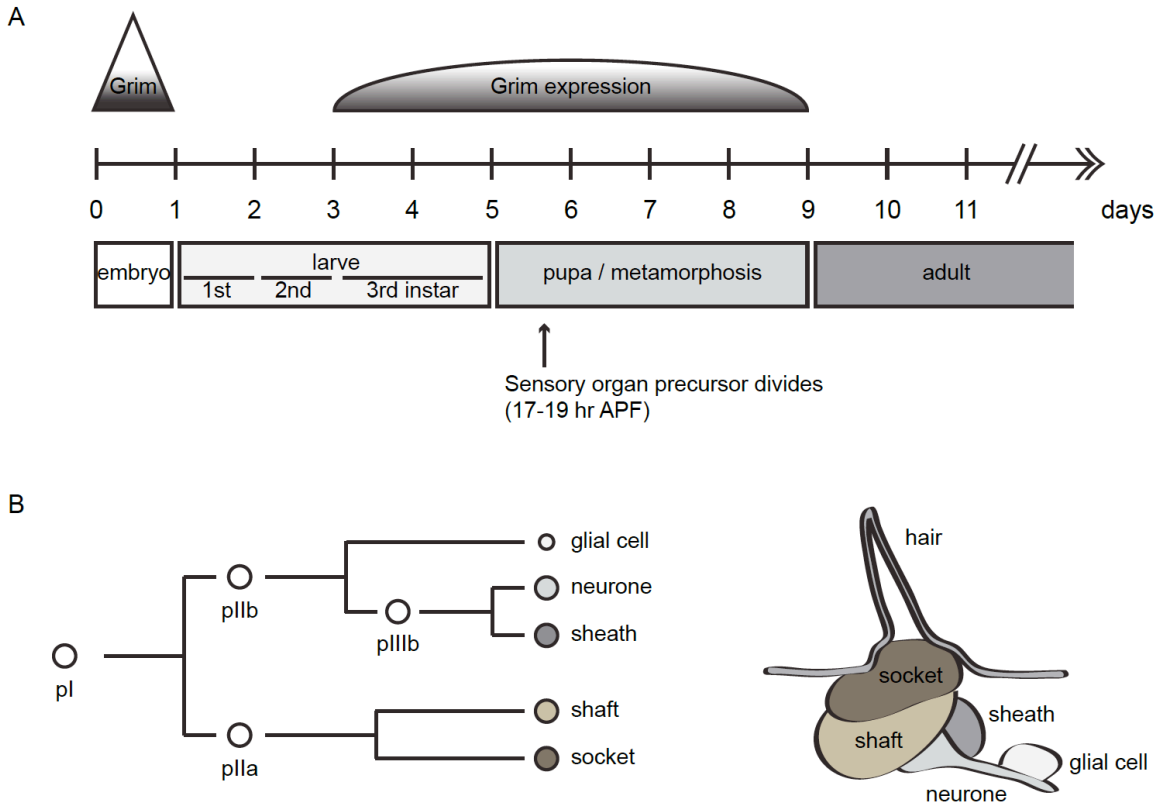

Figure 1.5 The expression of *grim* in *Drosophila* life cycle. (*A*) Flies have four main stages of the life cycle: embryo, larva, pupa, and adult. Grim mRNA transcripts were found mainly in embryo and pupa stage, where massive apoptosis is required for development. Sensory organ precursors start to divide 17-19 hr after puparium formation. (*B*) For the sensory organ precursor at the microchaete lineage, a glial cell is generated after asymmetric cell division. The glial cell will later be eliminated by the expression of Grim. At posterior wing margin, the pIIb cell undergoes Grim-dependent apoptosis, which gives rise to non-innervated mechanoreceptors. (The lower panel is adapted from Gho *et al*., 1999)

1.2.5 Grim

Three proapoptotic genes, located within the H99 locus, were identified: *reaper, grim, and hid* (Chen et al., 1996; Grether et al., 1995; White et al., 1994). There is a general lack of evidence regarding the specific inducers of *grim*, but Wingless reportedly activates *reaper*, *grim*, and *hid* (Lin et al., 2004), and the microRNA miR6 and miR11 inhibit the expression of *reaper, grim* and *hid* through their 3' UTR (Ge et al., 2012). UV or irradiation stimulates the expression of Reaper, but whether it increases the expression of Grim is unknown (Brodsky et al., 2000; Nordstrom et al., 1996). Similarly, it is unclear whether the hormone ecdysone upregulates *grim*, although it upregulates *reaper* and *hid* (Jiang et al., 2000). Due to the lack of a known specific inducer of Grim, we will utilize over-expressed Grim in *Drosophila* cells to study Grim-specific induced death. A metallothionein promoter under the control of copper will also be used to manipulate *grim* expression levels.

The mRNA transcripts of *grim* are observed during embryonic development with expression peaking at stage 13-15 (10-12h) (Fig. 1.5), and the expression is mainly located in the ventral nerve cord and in some macrophages (Chen et al., 1996). Loss of *grim* prevents neuroblast cell death during late embryogenesis, whereas loss of *reaper* has no such effect, indicating that Grim functions as a major cell death inducer in neuroblast cell death (Tan et al., 2011). To sculpt the adult central nervous system, programmed cell death is required to remove specific neurons that are no longer needed. Transcripts of *grim* are also observed in late 3rd instar larvae and during pupation, when massive apoptosis is required to shape the larva into the adult form (Fig. 1.5). During metamorphosis, *grim* is the main proapoptotic gene required for removing corazonin-

expressing peptidergic neurons in the ventral nerve cord, while *reaper* and *sickle* are less important during this process (Lee et al., 2013). During mid-pupal development, the sensory organ progenitor (SOP) cell undergoes asymmetric cell division and gives rise to five distinct cells (shaft, socket, sheath, neuron and glial cells) (Fig. 1.5) (Fichelson and Gho, 2003). In the thoracic microchaete bristle, a specialized sensory organ, the glial cell, will soon be eliminated by the existence of endogenous Grim (Wu et al., 2010). In the non-innervated bristle at the posterior wing margin, Grim and the dREAM/Myb-MuvB complex are responsible for apoptosis of the neuron and sheath cell precursor (Rovani et al., 2012) (Fig. 1.5).

With only 138 amino acids, Grim is predicted structurally to possess three alpha helices, named GH1, GH2, and GH3. GH1 begins immediately after the IBM; GH2 expands on the polyglutamine (polyQ)-rich region; and GH3 shares similarity with the GH3 domain of Reaper (Claveria et al., 2002) (Fig. 1.6). Grim is also able to induce cell death without its IBM, but the mechanism is still unclear. Immunocytochemistry data reveal that Grim is distributed in the cytoplasm but also forms punctae or ring structures (Claveria et al., 2002). Claveria and co-workers (2002) reported that the punctae colocalized with redistributed cytochrome *c* and caused mitochondria damage mainly through the GH3 domain even though the punctae did not colocalize with MitoTracker®. It remains unstudied whether the GH3 domain of Reaper and Grim share the same function or localization, and even if they do, the function of the GH3 domain of Reaper is still controversial. Interestingly, Grim also contains this polyglutamine expansion amongst all of the sequenced *Drosophila* species, but its function remains understudied. Notably, proteins with extensive polyQ repeats tend to aggregate and cause toxicity

(Shao and Diamond, 2007). Whether the polyQ repeats in Grim is under physiological or pathological range was never addressed, but to resolve this issue, we will use deletion mutants to test whether loss of the polyQ region will have effects on Grim-induced death.

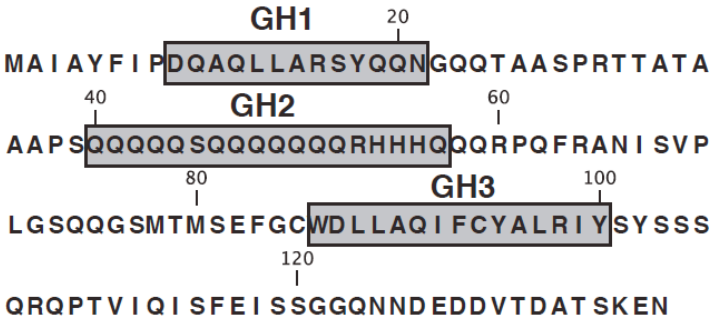

Fig. 1.6 The protein sequence of Grim in *D. melanogaster*. Grim contains 138 amino acids, and based on the structure prediction by Claveria *et al* (2002), it contains three alpha helices, named Grim Helix (GH) domains (highlighted in gray). The GH1 domain begins immediately after the IBM (amino acid 2-5); GH2 comprises a polyglutamine region; and GH3 contains a conserved sequence (amino acids 86-100). The C-terminus of Grim remains uncharacterized.

1.3 RNA granules, processing (P)-bodies, and translational repression

In response to different developmental stages or environmental cues, embryos and cells have different ways to regulate or reprogram the protein composition of a cell. One way is through translational control. Actively translated mRNA is circularized through an interaction between poly(A) binding protein (PABP) and the cap structure and is decorated with many ribosomes on a single mRNA (polyribosome, or polysome) (Fig. 1.7) (Besse and Ephrussi, 2008). Untranslated mRNA, along with different protein components, assembles into various mRNA granules in order to regulate the fate of the target mRNA. Such mRNA granules can be found in yeast, *C. elegans*, *Drosophila*, *Xenopus*, and mammals. They can be observed in the cytosol as nonmembranous punctae, including processing (P)-bodies, stress granules, neuronal granules, and germ cell granules (Buchan and Parker, 2009) (Fig. 1.7). Germ cell granules usually suppress maternal mRNA translation until mRNAs travel to the right position at right developmental stage. Neuronal granules contain mRNAs that need to be transported to dendrites for translation. Stress granules are enriched with stalled mRNAs and ribosomes due to environmental stress, such as heat shock. P-bodies are cytoplasmic mRNP granules of ~300-500 nm in diameter, where mRNA decay and translational repression occurs (Ernoult-Lange et al., 2012). The proteins found in P-bodies vary depending upon the cellular context and can be classified into several categories according to their functions, such as mRNA decapping (DCPs), enhancers of decapping (EDC3, EDC4), miRNA or siRNA function (Argonaute proteins), translational repression (Me31B, dFMR1, and GW182), and 5' to 3' degradation (Xrn1) (Garneau et al., 2007; Parker and Sheth, 2007). Depending upon the protein composition, mRNAs in a P-body can be

repressed, stored, degraded, or sent back to the translational machinery when needed. The proteins in P-bodies and stress granules can also shuttle back to the cytosol (Eulalio et al., 2007a).

Different mRNA granules share some common as well as unique protein components. Tia-1 (or Rox-8 in *Drosophila*) is an RNA-binding protein that localizes to stress granules in response to heat shock when translational initiation factor eIF2α is phosphorylated and translational initiation is halted (Kedersha et al., 2000). It is therefore used as a stress granule marker. Fragile X mental retardation protein (FMRP) is a dynamic RNA-binding protein and, depending upon cell types or conditions, it interacts with the cargo mRNA in the nucleus, binds to the coding region of mRNA, and stalls ribosomes (Kim et al., 2009). It also coordinates with the P-body component Me31B to negatively regulate dendritic growth in sensory neurons (Barbee et al., 2006; Darnell et al., 2011). Decapping enzymes, such as DCP2 and DCP1, are found mainly in P-bodies, while ribosomal proteins and initiation factor 4E (eIF4E) are usually absent in P-bodies (Buchan and Parker, 2009; Eulalio et al., 2007a; Parker and Sheth, 2007). P-bodies can be dynamic as well as stationary granules, and the size of which varies depending upon the untranslated mRNA pool (Aizer and Shav-Tal, 2008; Teixeira et al., 2005). The translational repressor cycloheximide traps mRNAs in polysomes leading to a decrease in the visible cytoplasmic pools of P-bodies in the cell. Puromycin induces the premature release of mRNA to the cytosol, such that it increases both P-body size and number. It is believed that these mRNA granules are important sites for mRNA storage, miRNA function, mRNA decay, and translational repression, but whether P-bodies are a

consequence or a cause of accumulated of untranslated mRNA remains controversial (Eulalio et al., 2007b).

Recent studies have found that 50 to 70% of P-bodies dynamically associate with mitochondria, and that while disruption of mitochondria does not affect the size or localization of the P-bodies, it does affect miRNA-mediated RNA interference (Huang et al., 2011). It is currently unclear whether stress granules also associate with mitochondria; due to their larger size (1-2 μm), it is difficult to determine whether they are randomly distributed in the cytosol or around the mitochondria (Ernoult-Lange et al., 2012).

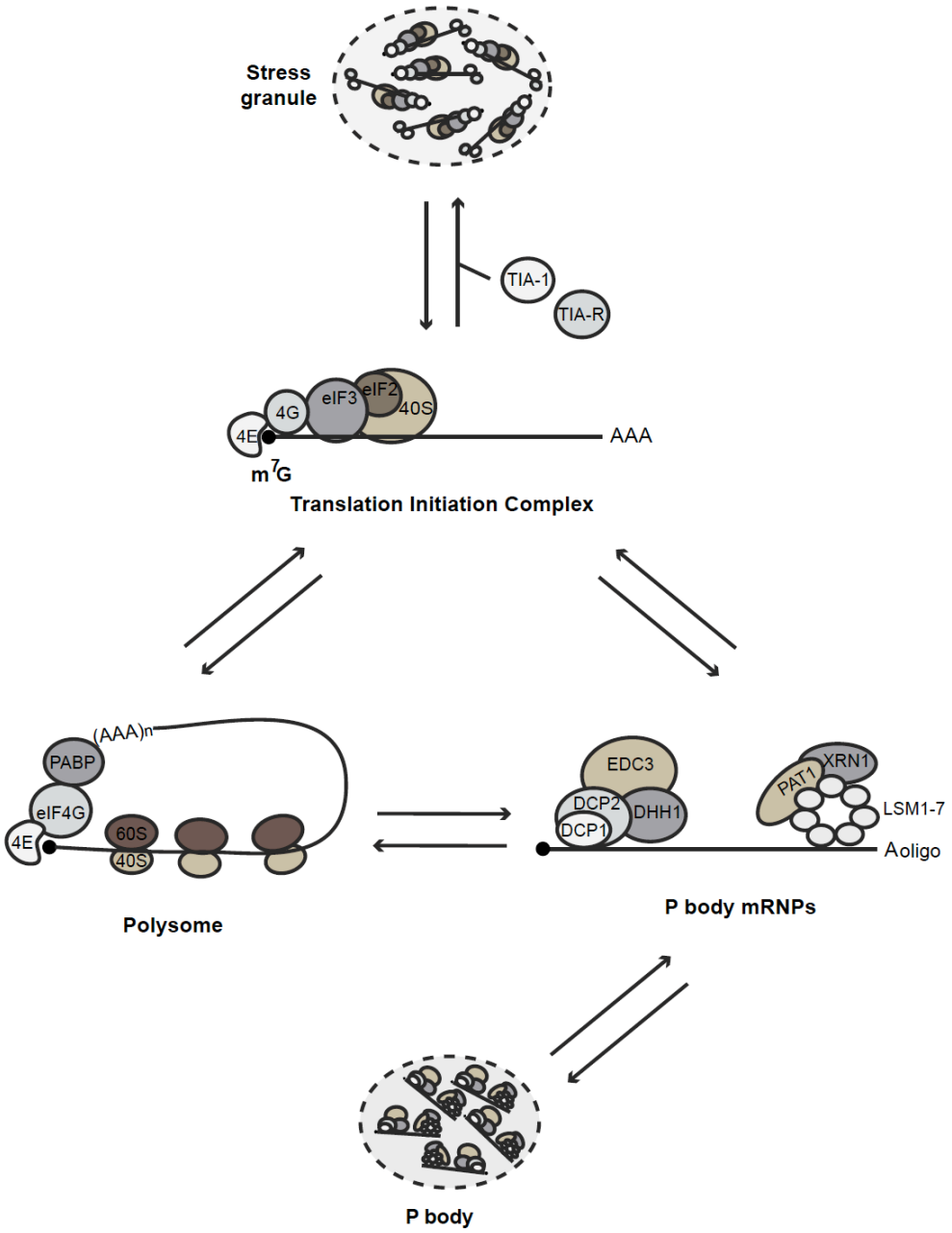

Fig. 1.7 The mRNA cycle. After mature mRNA is exported to the cytosol, eIF4E binds the cap of mRNA and the translational initiation complex is recruited. Translation is facilitated by mRNA circularization through the interaction between PABP and eIF4G, allowing many ribosomes to translate one mRNA molecule at the same time (polysome). Stress granules assemble during cellular stress causing accumulation of stalled mRNA and TIA-1/TIAR complexes. The P-body is a storage site in the cytosol for untranslated mRNA transcripts. Decapping enzymes, enhancers of decapping proteins, or exonucleases (LSM complexes) are enriched in P-bodies to regulate mRNA stability. (Modified from Parker and Sheth, 2007; Besse and Ephrussi, 2008)

1.3.1 Decapping enzymes

A eukaryotic mature mRNA contains a cap structure at its 5'-end and a polyadenylated tail at its 3'-end, which facilitates its translation and also protects it from degradation by 3' →5' exonucleases. Decapping is a critical step in eukaryotic mRNA decay, after which, 5' →3' exonucleases can degrade mRNA. An mRNA molecule can also be degraded from the 3'-end after deadenylase removes the poly(A) tail (Garneau et al., 2007). DCP2 is the main decapping enzyme, while DCP1 and EDC3 are enhancers of decapping. DCP2 has closed and open conformations, and when interacting with DCP1, the closed form is stabilized, resulting in higher decapping activity (She et al., 2008). The crystal structure of the yeast DCP2-DCP1 complex reveals that the interaction interface is not highly conserved in higher organisms (She et al., 2008), which explains why extra molecules, such as EDC3, RCK/p54 (Me31B homolog in human), and EDC4/Ge-1/Hedls, are required to stimulate decapping activity in higher organisms (Bail and Kiledjian, 2006; Chang et al., 2014). The C-terminus of DCP1 has a unique asymmetric trimerization domain that forms a kink that is structurally required for the assembly of active decapping complexes through interactions with DCP2 and EDC4 (Tritschler et al., 2009) (Fig. 1.8).

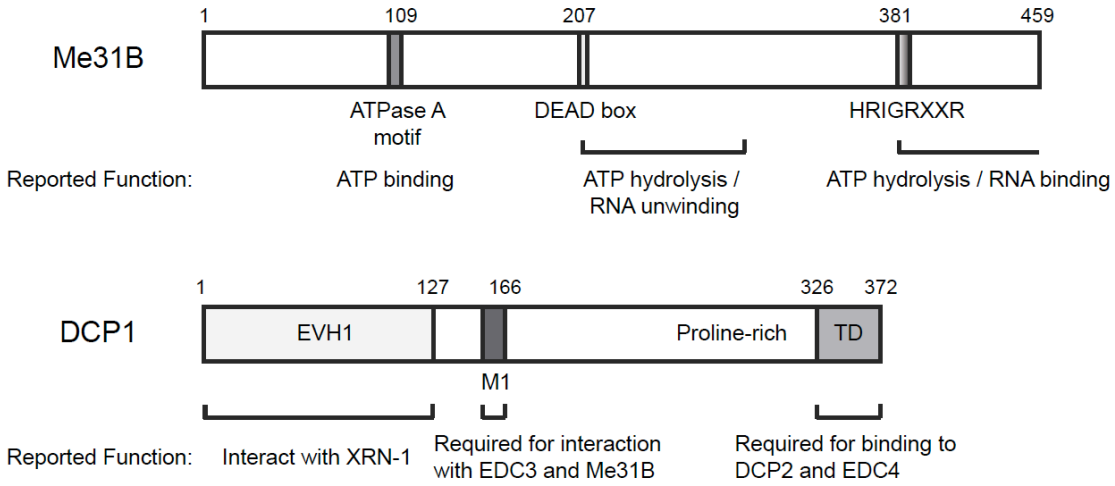

Fig. 1.8 The functional and domain analysis of Me31B and DCP1. Me31B belongs to the DEAD-helicase protein family, which contains several characteristic motifs: the ATPase A motif, the DEAD box, and the HRIGRXXR region. They assemble into two important domains (N and C domains) that are important for ATP binding, ATP hydrolysis, RNA binding, or RNA unwinding. DCP1 interacts with and stabilizes DCP2 through its unique C-terminal trimerization domain (TD). It also has motif 1 that is required for interaction with enhancer of decapping (EDC3) and Me31B. The N-terminal EVH1 domain directly interacts with XRN-1, which is important for exonuclease activity.

1.3.2 The maternal expression at 31B (Me31B)

In the process of *Drosophila* oogenesis, a large amount of mRNA and proteins are synthesized by nurse cells and transported to the oocyte for establishing the polarity of the future embryo. During the transport, mRNA transcripts are translationally repressed by protein complexes that include maternal expression at 31B (Me31B), so named because the gene is located on the chromosome 31B region and has high expression levels in oocytes (de Valoir et al., 1991; Nakamura et al., 2001). Even though Me31B is not required for transporting mRNA, it is important for silencing mRNA translation during oocyte development (Nakamura et al., 2001). Me31B draws a lot of attention because it belongs to a highly conserved protein family of ATP-dependent DEAD-box helicase proteins that regulates biological function through modifying RNA-protein complexes (Fig. 1.8). Me31B and all its homologs, Dhh1p (*Saccharomyces cerevisiae*), CGH-1 (*Caenorhabditis elegans*), Xp54/DXX6 (*Xenopus laevis*), and RCK/p54 (mouse and human) are translational repressors, and many of them are important during oogenesis, early embryo development, and in regulating mRNA expression in cells or dendrites (Akao et al., 1995; Coller et al., 2001; Navarro et al., 2001; Smillie and Sommerville, 2002).

Other than translational repression, Me31B and its homologs interact with decapping proteins to enhance mRNA decapping. They also associate with subunits in the deadenylase complex (Coller et al., 2001), and RCK/p54 interacts with Argonaute (Ago) proteins *in vivo*. Depletion of RCK/p54 affects Ago2 localization, but not Ago2 function (Chu and Rana, 2006). Interestingly, RCK/p54 expresses at high levels in many malignant cancer cell lines and human colorectal adenocarcinoma patient samples. RCK

promotes cell proliferation when overexpressed and is downregulated during differentiation through uncharacterized mechanisms (Akao et al., 2006; Nakagawa et al., 1999).

How translation is inhibited by Me31B is not fully understood, but mutation in the DEAD-box or HRIGR domains activate translation in reporter assays (Minshall and Standart, 2004). The crystal structure of Dhh1p reveals that the protein contains two domains, an N-domain and a C-domain, which interact with each other to form a large channel. The N-domain is important for ATP binding and hydrolysis, and the C-domain is important for RNA binding. ATP binding is not required for RNA binding, but it is important for conformational changes and the function of Dhh1p (Cheng et al., 2005). It is difficult to demonstrate the helicase activity of Me31B or its homolog *in vitro* due to technical issues or a lack of necessary cofactors; so most studies have examined the function of whole Me31B complexes. Me31B interacts with *Drosophila* fragile X mental retardation protein (dFMR1) and forms a complex with Staufen to regulate dendrite morphogenesis in sensory neurons (Barbee et al., 2006; Hillebrand et al., 2007). Me31B overexpression reduces dendritic complexity, while loss of Me31B increases dendritic spine number and length (Hillebrand et al., 2010). Its interaction with Tral or EDC3 is mutually exclusive, and it forms complexes with DCP1 and CUP when interacting with Tral. A different complex is formed with DCP1 and DCP2 when associating with EDC3 (Tritschler et al., 2008). It is possible that Me31B performs different functions through the recruitment of different protein components, but confirmation of this will require future studies.

1.4 Dissertation objectives

Apoptosis is a highly regulated process involving proteolytic cascades that lead to activation of caspases, which in turn cause cell self-destruction. This evolutionarily conserved pathway is required during development and morphogenesis of metazoans, and deregulation of apoptosis can result in human diseases, such as cancer, neurodegeneration and autoimmune disorders. *Drosophila* has been an excellent model system in which to study apoptosis due to its conservation and similarities with mammalian intrinsic pathways. In flies and mammals, cell death can be regulated by altering the balance among caspases, IAPs, and IAP antagonists. IAPs prevent caspase-mediated cell death not only through direct binding and inhibition, but also by directly ubiquitinating caspases, which inactivates them or targets them for degradation (Ditzel et al., 2008; Lee et al., 2011; Li et al., 2011; Muro et al., 2002; Wilson et al., 2002; Yan et al., 2004). IAP antagonists promote cell death by displacing IAP-bound caspases as well as by promoting IAP autoubiquitination (Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). While the roles of caspases in mediating cell death in multicellular organisms have been studied for the past two decades, it is still unclear how cell death activators/enhancers, such as IAP antagonists, are regulated at the molecular level. Our goal was to study the regulation of IAP antagonist-induced cell death. We used the *Drosophila* IAP antagonist Grim as an example to investigate IBM-dependent and IBM-independent cell death, since some IAP antagonists are known to induce cell death with a second mechanism. We discovered that Grim is a target for caspase-mediated cleavage at Grim's C-terminus, but the detailed regulation and significance was not clear. **In chapter 3, we hypothesized that cleavage of Grim results in a truncated form of Grim that cannot be turned**

over and thus results in a feed-forward caspase amplification loop. We showed a novel regulation between caspases and the proteasome system in that Grim is turned over through DIAP1-mediated ubiquitination and cleavage of Grim at its C-terminus rescued it from degradation, markedly increasing Grim-induced cell death.

In addition to IBM-dependent cell death, Grim can induce cell death in an IBM-independent manner, but the mechanism remains unclear, particularly with regard to where in the cell that Grim mediates this effect (Chen et al., 2004; Claveria et al., 2002). Previous studies suggest Grim partially colocalizes with mitochondria (Claveria et al., 2002), but we discovered that it instead localizes to P-bodies. **In Chapter 4, we hypothesized that Grim mediated cell death, at least in part, through its association with P-bodies and the induction of translational repression.** We demonstrated that the C-terminus of Grim was required for both its localization to P-bodies and its killing activity. Knockdown of Me31b, a translational repressor, can significantly reduced IBM-independent cell death. **Overall, we aim to understand at a cellular level how Grim-induced cell death is executed and regulated through both IBM-dependent and –independent mechanisms.**

CHAPTER 2

Materials and methods

2.1 Reagents and antibodies

Maxfect™ (#TR-1000) transfection reagent was purchased from KD Medical (Columbia, MD). MG-132, cycloheximide, zVAD-fmk, and Ac-DEVD-AMC were obtained from Biomol-Enzo Life Sciences. MitoTracker® Red CM-H_2Xros (M-7513) was purchased from Life Technologies. Antibody against GFP (CS-2956), HA (CS-2367), myc (CS-2276), ubiquitin (CS-3936), K48-specific ubiquitin chains (CS-8081), and K63-specific ubiquitin chains (CS-5621) were purchased from Cell Signaling Technology. HSV antibody was obtained from both Novagen (69171) and Novus (NB120-9533), while Grim antibody (sc-1573) was purchased from Santa Cruz Biotechnology. Tubulin antibody (E7) was obtained from the Developmental Studies Hybridoma Bank, and Flag antibody (F1804 and F2555) was purchased from Sigma-Aldrich. DIAP1 antibody was a gift from Dr. Kristin White (Massachusetts General Hospital/Harvard Medical School, Charlestown, MA). Antibodies to DrICE and DRONC were raised in house.

2.2 Cell culture and transfection

Drosophila S2 cells were grown and maintained in HyClone SFX-Insect medium (Thermo Scientific, Logan, UT) at 25ºC. For transfection, cells were plated ($1x10^6$ cells/well) overnight in a 12 well plate. Then, 1 μg of total DNA and 4 μl Maxfect™

reagent were diluted in separate tubes with 100 µl of insect medium resting at room temperature for 10 mins. Diluted DNA was then added into the transfection reagent tube and incubated at room temperature for another 10 mins. The final mixture was applied onto cells gently.

2.3 Bacterial and fly expression constructs

C-terminal His_6-tagged inactive DrICE (C211A) was cloned into pET21 vector (Novagen) with NcoI-XhoI sites. N-terminal His_6-tagged inactive Dronc (C318A) was cloned into pET28 vector (Novagen) with BamHI-NotI sites. Grim constructs were cloned into a pIE vector (Novagen) with an N-terminal Flag tag by using SacII-NotI sites or into a pRmHa3 vector with a C-terminal HSV-His_6 tag by using EcoRI-NotI site. DIAP1 and DIAP2 constructs were cloned into a pIE vector with an N-terminal HA or GFP tag by using SacII-NotI sites. Specific point mutation was introduced into plasmid DNA using site-directed mutagenesis. Ubiquitin was cloned into a pMT vector (Invitrogen) using KpnI-NotI sites with an N-terminal Flag tag. UbcD1 and Morgue constructs were cloned into a pIE vector with an N-terminal myc tag using SacII-NotI sites. pCasper-mitoGFP and pAGW-dMFN constructs were gifts from Dr. Kristen White (Abdelwahid et al., 2007) and Dr. Sally Kornbluth (Thomenius et al., 2011) respectively.

For various GFP-Grim or Grm fragments, GFP was first cloned into SacII and NotI sites into a pIE vector (pIE-GFP). BamHI and XhoI site were introduced by using a downstream primer. Grim and its mutants were then cloned separately into BamHI-XhoI sites. pIE-mCherry construct was made by cloning mCherry into SacII and BamHI site in a pIE-GFP vector. For various mCherry-Me31B or its deletion mutants, Me31B or its

deletion mutants was cloned into XhoI-ApaI sites. DCP1 construct was cloned into a pIE vector with an N-terminal DsRed by using SacII-ApaI sites. Rox8 construct was cloned into BamHI-XhoI sites of a pIE-GFP construct. dFMR1 was cloned into a pIE-mCherry construct using BamHI-XhoI sites.

2.4 Cell lysate preparation

After transfection or treatments, cells were spun down at 1,000 × g for 5 mins. Cells were lysed in NP40 lysis buffer (20 mM Tris HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 10 mg/mL leupeptin, 10 mg/mL pepstatin, 10 mg/mL aprotinin, 200 mM PMSF) on ice for 10 min and spun at 18,000 × g for 10 min at 4ºC. For whole cell lysate preparation, 2X loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added to the cell pellet and incubated at 95ºC for 5 min. Genomic DNA was sheared by sonication, and samples were subjected to SDS-PAGE/immunoblotting.

2.5 Immunoprecipitation and immunoblotting

Cells were lysed in NP40 lysis buffer on ice for 10 min and supernatant was collected after 10 min spin at 18,000 × g. Protein concentration was determined using Bradford assay (Bio-Rad), and 1 mg of lysate was used for immunoprecipitation. Lysates were incubated with 1 µl rabbit anti-HSV antibody and 30 µl protein agarose G beads (GE Healthcare Life Sciences, Piscataway, NJ) for 2 h at 4ºC. Beads were then washed (4X) with lysis buffer and analyzed by SDS-PAGE/immunoblotting.

2.6 Immunostaining

Drosophila S2 cells were transfected with pRmHa3-Grim(D132A)-HSV in the presence of zVAD-fmk. After 8h induction, cells were incubated with MitoTracker® Red prior to 4% paraformaldehyde fixation, and washed with PBS three times. Cells were then permeabilized with 0.1% Triton-X 100 and blocked with 1% (w/v) bovine serum albumin for 1h, followed by incubating with anti-HSV antibody (1:1000 dilution). After washing with PBS three times, cells were incubated with fluorophore-conjugated secondary antibody (1:1500 dilution) for 1hr at room temp. DNA was stained using Hoechst 33342 (1 μg/ml). Images were taken using Nikon microscopy system (TE2000) and NIS-Elements Ar Micorscope Imaging Software.

2.7 Ubiquitination assay

Drosophila S2 cells were transfected with the indicated Grim and DIAP1 constructs (500 ng each). Following transfection for 24 h, cells were induced with $CuSO_4$ for 8 h and lysed under denaturing conditions (6 M guanidine-HCl, 0.1 M NaH_2PO_4, 20 mM Tris-HCl, pH 8.0, 10 mM N-ethylmaleimide and protease inhibitors). His-tagged proteins were pulled down after incubating the samples with Ni-NTA beads (Qiagen) for 2 hr under denaturing conditions. Beads were then washed with 25 mM imidazole buffer (0.5 M NaCl, 50 mM Tris-HCl, pH 8.0, 25 mM imidazole) and heated for 5 min at 95ºC after adding a loading dye (2X). Samples were analyzed by SDS-PAGE/western blotting.

To examine ubiquitination of Grim in the absence of ectopically expressed DIAP1 or DIAP2, cells were transfected with pRmHa3-Grim (1μg) and pMT-Flag-ubiquitin (0.2

μg). After 24h transfection, cells were induced with $CuSO_4$ for 8h in the presence of zVAD-fmk and MG132. Cells were then lysed in 1% SDS and boiled at 95 °C for 10 min and centrifuged to remove any pellet. The supernatant was then diluted with NP-40 lysis buffer and incubated with Flag antibody and protein G sepharose beads at 4 °C for 2h. Finally, beads were washed with lysis buffer and analyzed by SDS-PAGE/immunoblotting.

2.8 RNAi in S2 cells

Double-stranded (ds) RNA was synthesized using MEGAscript T7 *in vitro* transcription kit (Life Technologies) and annealed at 65ºC for 10 min. S2 cells were then treated with 40 nM dsRNA for 24 h. Sequence-specific primers with the T7 promoter sequence (GAATTAATACGACTCACTATAGGGAGA) and indicated gene-specific sequences were used (Table 2.1).

Table 2.1 RNAi primers used in this study

drice	Forward primer (F): GTACAACATGCGCCACAAG Reverse primer (R): CAGGAACCGTCGAGTAGG
dronc	F:GACAATGGTGACCTCCTCGT R:GCCATCACTTGGCAAAACTT
ubcd1	F: GGCGTTAAAAAGAATCAATAAGGA R: CATAGCATACTTTCTAGTCCACT
morgue	F: TTCATATACTTCCCGGAGCGA R: TCACTAGCGCGGTGCAATTAG
gfp (control)	F: GAGTGAGCAAGGGCGAGGAGC R: GATTGTACAGCTCGTCCATGCC

Table 2.1 (cont.)

tnf (control)	F: CCCAGGGACCTCTCTCTAATCAGC R: GCAATGATCCCAAAGTAGACCTGC
diap1	F: GTTGTGCAAGATCTGCTACGG R: TAACTGGCAGGCTTTGTGATCT
diap1 3'UTR	F: TAACCCCAATGCGCACCCAA R: TTTTCGGTAATATATTAGTTTTATT
me31b	F: GCCACTCCCGGACGAATATTA R: AGATTGCGACAGAGTCCTTGG
dcp1	F: TGGCCGACGAGAGCATCACGC R: TCCCTGATCCTTGGACTTGAG
dcp2	F: GAGACCGGGTTCGATATCAC R: CGATCCCGCTCCCGTTCCA
gawky (GW182)	F: CGGGAGGTATAGGAATAGCCG R: ATTGCTTGCTTTGCTTAATGATCT

2.9 qPCR (real-time PCR) and RT-PCR

Total RNA was isolated and treated with DNase to prevent any possible contamination from plasmid DNA and cleaned up using RNeasy Mini kit (Qiagen). cDNA template was synthesized by using random primers. Real-time PCR was performed on Applied Biosystems 7900HT. Gene specific primers were used to detect each gene (Table 2.2). The resulting Ct values were applied to standard curves generated for both *diap1* and *grim*. Results are presented as the number of mRNA copies present in 40 ng of total RNA.

Table 2.2 RT-PCR and qPCR primers

grim	F: TTCCGTGCCAATATTTCCGTG R: GTC CTCATCGTTGTTCTGACC
diap1	F: AGAGCTCTTCGATTGGAGGG R: GTATGGAGGTGGAGCCGCT
gapdh	F: GCAAGCAAGCCGATAGATAAAC R: CAACGGTGCCCTTAAAACGTC
dcp1	F: GGCAGGGACAATCACGAGAG R: CAGGTAGGCCTTGTGCAGCT
dcp2	F: TCCAACACATTCCCTTCCTGAA R: GGAACCAGTCGCAGCACTTG
me31b	F: CCGATATGTCGCATTGCAGAAT R: TAATAGCAATACCCAAATGACCG
gw182	F: TGTCCTTTCCTCATAATAACCTTA R: CCTGGAGATGCTTAATATTGCTA

2.10 Pulse-chase experiment

After a 24 h transfection, Grim expression was initially induced in S2 cells with $CuSO_4$ for 2 h. Cells were then washed once with PBS and incubated with cycloheximide (10 μg/ml) for 0-8 h. All pulse-chase experiments were performed in the presence of zVAD-fmk (50 μM) in order to prevent cell death. Whole cell lysates were collected and analyzed by SDS-PAGE/western blotting.

2.11 DEVDase activity assay

Cells were lysed in buffer (10 mM Tri-HCl, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton-X 100) for 10 min on ice and spun at 18,000 × g for 10 min. Supernatant was normalized to equal protein concentration (4-5 mg/mL) and 80 µL of lysates were incubated with an equal volume of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT) containing the fluorescent substrate, Ac-DEVD-AMC (final concentration = 25 µM). DEVDase activities were immediately measured (Ex: 354 nm, Em: 442nm) in a 96-well pate using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

2.12 Caspase cleavage assay

S2 cells were cotransfected with HA-DIAP1 and either wild-type Grim, Grim (D132A), or Grim (K136R). After 24 h, cells were incubated with $CuSO_4$ (0.7 mM) for an additional 8 h in the presence of zVAD-fmk (50 µM) and MG132 (10 µM) to prevent caspase activity and proteasomal-dependent turnover of proteins. After washing the cells, lysates were prepared and incubated for 30 min at 37ºC, with or without recombinant DrICE (10 µg), in a total volume of 100 µL. Protein loading buffer (5X) was then added to terminate the reaction, and the samples were analyzed by SDS-PAGE/immunoblotting.

2.13 Recombinant protein expression and antibody generation

All recombinant proteins were expressed in *E. coli* strain BL21(DE)pLysS with N-terminal or C-terminal His_6 tag. Bacteria were grown in 400 ml LB culture till $O.D._{600}$ reaches about 0.4-0.6. Protein expression was then induced with 1 mM IPTG at 28ºC

for 4h. Bacteria were collected by centrifugation and resuspened in 40 ml of lysis buffer with protease inhibitors (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, 1mM PMSF, 2 μg/ml aprotonin, 2 μg/ml pepstin, 2 μg/ml leupeptin, 1mM DTT, pH8.0). After DNA was sheared by sonication, lysates were centrifuged at 20,000 × g for 30mins. Recombinant protein was purified on an FPLC (GE Healthcare Life Sciences, Piscataway, NJ) coupled to a column packed with Ni^{2+}-NTA beads (Qiagen Sciences, Germantown, MD). For antibody generation, protein was further dialyzed with PBS and used to immunize rabbits (Washington Biotechnology, Inc., Baltimore, MD). Affinity purified antibodies were diluted 1:500,000 prior to immunoblotting.

2.14 DIC microscope for tracking dead cells

S2 cells were transfected with various Grim mutant constructs and GFP-DIAP1. After 24h transfection, cells were replated on Lab-Tek II chamber slides, incubated with $CuSO_4$, and imaged every hour for 16 h by time-lapse DIC microscopy (Nikon TE2000). Only GFP positive cells were counted. Dead cells were tracked and quantified based on cell membrane blebbing. The cell death percentage was calculated by dividing the dead cells by the GFP positive cells.

2.15 Loss of GFP assay

S2 cells were transfected with 800ng pRmHa3-Grim-HSV or control vector along with 100ng pAc-GFP. After 16 h, cells were split in half; one portion was induced with $CuSO_4$, while the other was not. GFP positive cells were counted after 6 h or 24 h induction by flow cytometry (BD Accuri™ C6 flow cytometer). The percent of GFP

positive cells was used to represent cell survival rate, and was calculated by 100% x (GFP^+ cells in the copper-treated sample) / (GFP^+ cells in the non copper-treated sample).

CHAPTER3

Caspase-dependent regulation of the ubiquitin-proteasome system through direct substrate targeting

The data described in this chapter have been published in *The Proceedings of the National Academy of Sciences* under Dr. Bratton's supervision (PMID: 23940367).

3.1 Summary

Drosophila inhibitor of apoptosis (IAP) 1 (DIAP1) is an E3 ubiquitin ligase that regulates apoptosis in flies, in large part through direct inhibition and/or ubiquitination of caspases. IAP antagonists, such as Reaper, Hid, and Grim, are thought to induce cell death by displacing active caspases from baculovirus IAP repeat domains in DIAP1, but can themselves become targets of DIAP1-mediated ubiquitination. Herein, we demonstrate that Grim self-associates in cells and is ubiquitinated by DIAP1 at Lys136 in an UbcD1-dependent manner, resulting in its rapid turnover. K48-linked ubiquitin chains are added almost exclusively to BIR2-bound Grim as a result of its structural proximity to DIAP1's RING domain. However, active caspases can simultaneously cleave Grim at Asp132, removing the lysine necessary for ubiquitination as well as any existing ubiquitin conjugates. Cleavage therefore enhances the stability of Grim and initiates a feed-forward caspase amplification loop, resulting in greater cell death. In summary,

Grim is a caspase substrate whose cleavage promotes apoptosis by limiting, in a target-specific fashion, its ubiquitination and turnover by the proteasome.

3.2 Introduction

Apoptosis, or programmed cell death, is broadly conserved throughout nature, from flies to humans (Kornbluth and White, 2005). In most instances, the execution of apoptosis is carried out by cysteinyl aspartate-specific proteases (i.e., caspases) through proteolytic-based signal transduction pathways (Fuentes-Prior and Salvesen, 2004). Upstream initiator caspases, such as caspase-9 in humans and its homologue Drosophila Nedd2-like caspase (DRONC) in flies, are first activated via their interactions with adapter proteins and in turn activate the downstream effector caspases, caspase-3 and Drosophila interleukin-1β–converting enzyme (DrICE), respectively (Fuentes-Prior and Salvesen, 2004; Kumar, 2007). Once activated, effector caspases are responsible for dismantling the cell through cleavage of literally hundreds of structural and regulatory proteins (Luthi and Martin, 2007). Caspase cleavage can inactivate proteins or generate dominant-negative inhibitors, as in the case of gelsolin, RIP1, and eIF4E-BP1 (Luthi and Martin, 2007). Moreover, caspase cleavage of numerous substrates, including IRF-3, ErbB2, cyclin E, claspin, SSRP1, and Twist, can enhance their turnover by the proteasome (Demontis et al., 2006; Landais et al., 2006; Plesca et al., 2008; Sears et al., 2011; Semple et al., 2007; Tikhomirov and Carpenter, 2001). Conversely, caspases can also constitutively activate proteins, particularly kinases such as PKC and Mst1 (Emoto et al., 1995; Graves et al., 1998), or change the function of a protein altogether, as seen in the conversion of antiapoptotic BCL-2 proteins into proapoptotic BAX-like proteins (Cheng et al., 1997).

Notably, even following the activation of caspases, inhibitor of apoptosis (IAP) proteins, such as X-linked IAP (XIAP) in mammals and DIAP1 in flies, can suppress apoptosis through inhibition of caspases (Chai et al., 2003; Deveraux et al., 1997; Meier et al., 2000; Riedl et al., 2001; Shiozaki et al., 2003; Yan et al., 2004). All IAPs contain baculovirus IAP repeat (BIR) domains and many possess RING and UBA domains, imparting them with E3 ubiquitin and NEDD8 ligase activity and the ability to bind polyubiquitin chains (Gyrd-Hansen and Meier, 2010; Salvesen and Duckett, 2002). Thus, XIAP and DIAP1 directly bind and inhibit, ubiquitinate, and/or neddylate initiator and effector caspases through distinct BIR domains (Broemer et al., 2010; Deveraux et al., 1999; Riedl et al., 2001; Shiozaki et al., 2003; Wilson et al., 2002; Yan et al., 2004). In some circumstances, ubiquitination marks these enzymes for proteasomal degradation, whereas, in other cases, K63-based ubiquitination or neddylation fail to increase protein turnover but nevertheless inhibit protease activity through as yet ill-defined mechanisms (Broemer et al., 2010; Ditzel et al., 2008; Lee et al., 2011; Tenev et al., 2005).

Finally, a further level of regulation exists in the form of endogenous inhibitors of IAPs. These so-called "IAP antagonists" possess an IAP binding motif (IBM) through which they bind to IAPs and disrupt their interactions with caspases (Wu et al., 2001). Reaper, Hid, and Grim were the first IAP antagonists to be discovered in flies and were shown to regulate cell death during development, at least in part, by binding to DIAP1, displacing caspases, and inducing autoubiquitination and turnover of DIAP1 (Chai et al., 2003; Goyal et al., 2000; Holley et al., 2002; Lisi et al., 2000; Ryoo et al., 2002; Wang et al., 1999; Wing et al., 2002; Yan et al., 2004; Yokokura et al., 2004). In the present study, we have discovered that DIAP1, in conjunction with the E2 ubiquitin-conjugating

enzyme UbcD1, polyubiquitinates Grim through K48- but not K63-based linkages, resulting in increased Grim turnover. Grim self-associates in cells and binds to both the BIR1 and BIR2 domains in DIAP1, but only the BIR2-bound Grim is significantly ubiquitinated by DIAP1 in a RING-dependent manner. More surprisingly, Grim is also cleaved by caspases at its C-terminus, removing the only lysine residue present in this IAP antagonist. Following caspase cleavage, Grim still binds to DIAP1 but is no longer ubiquitinated and therefore persists in cells, propagating the death signal through increased activation of caspases.

3.3 Results

3.3.1 Grim is cleaved by caspases at Asp132 during apoptosis

In an experiment designed to characterize the putative IBM-independent effects of Grim, Grim was cloned with an N-terminal Flag tag to block access to its IBM (Claveria et al., 2002). The tag however was initially cloned in-frame with the normal initiator methionine in Grim (i.e., Flag-MAIAY-Grim), and when expressed, two bands were unexpectedly observed (Fig. 3.1*A*, lane 3). Importantly, the lower band disappeared in cells pretreated with the polycaspase inhibitor zVAD-fmk, so that only the upper band was observed (Fig. 3.1*A*, lane 4). As we immunoblotted for Grim by using antibodies to the N-terminal Flag tag, this strongly suggested that Grim was cleaved at its C-terminus by a caspase, and, indeed, endogenous DrICE was activated by Flag-MAIAY-Grim and inhibited by zVAD-fmk (Fig. 3.1*A*, lanes 3 and 4).

Given that Grim requires its exposed IBM to displace active caspases from DIAP1, we questioned whether the Flag-MAIAY-Grim construct might have undergone

alternative translation from two different start sites, so that untagged MAIAY-Grim was produced in addition to Flag-MAIAY-Grim (Fig. 3.1*B*). We reasoned that if IBM-competent AIAY-Grim activated caspases, they might in turn cleave the C-terminus of Flag-MAIAY-Grim, yielding two Flag-tagged products (Fig. 3.1*B*). To test this prediction, we first removed the internal methionine from our Flag-MAIAY-Grim construct and transfected cells with Flag-AIAY-Grim. As anticipated, expression of Flag-AIAY-Grim resulted in a single translated product that failed to activate caspases or undergo cleavage (Fig. 3.1*A*, compare lanes 1 and 3). Second, we transfected cells with Flag-AIAY-Grim and exposed them to the translation inhibitor cycloheximide (CHX), a known inducer of apoptosis in S2 cells (Fraser et al., 1997). As expected, CHX induced (and zVAD-fmk inhibited) the activation of DrICE and the subsequent cleavage of Flag-AIAY-Grim at its C-terminus (Fig. 3.1*C*, lanes 3 and 4).

Having demonstrated that Grim is cleaved by caspases, we next sought to identify the caspases responsible for cleavage. Grim is thought to promote DARK-DRONC apoptosome-mediated activation of effector caspases, such as DrICE, by antagonizing DIAP1-dependent turnover of DRONC. Therefore, we knocked down the expression of DRONC or DrICE in S2 cells by RNAi and found that depletion of either caspase inhibited the cleavage of Flag-MAIAY-Grim (Fig. 3.1*D*). Notably, whereas knockdowns of DrICE and particularly DRONC appeared to be near-complete, zVAD-fmk inhibited Grim cleavage more efficiently than knockdown of either caspase. Thus, we cannot formally exclude the possibility that other caspases are involved in the cleavage of Grim.

Finally, to map the caspase cleavage site(s) in Grim, we examined its C-terminus and noted the presence of two potential DXXD motifs, DEDD129 and DVTD132, both

of which were followed by small uncharged amino acids (valine and alanine, respectively) in the P1′ position (Fig. 3.1*B*). We suspected the cleavage site was DVTD132↓A because of the more preferable P2 amino acid, but feared that mutation of Asp132 to alanine might redirect some cleavage to Asp129. Therefore, we incorporated a single D132A or a compound D129A/D132A mutation into the Flag-MAIAY-Grim construct and expressed the mutants in S2 cells. As shown in Fig. 3.1*E*, mutation of Asp132 alone inhibited the C-terminal cleavage of Grim (Fig. 3.1*E*, compare lane 3 with lanes 1 and 2). Thus, Grim (via its IBM) antagonizes DIAP1 and activates caspases, which in turn cleave Grim at Asp132 to liberate its C-terminus (Fig. 3.1*B*).

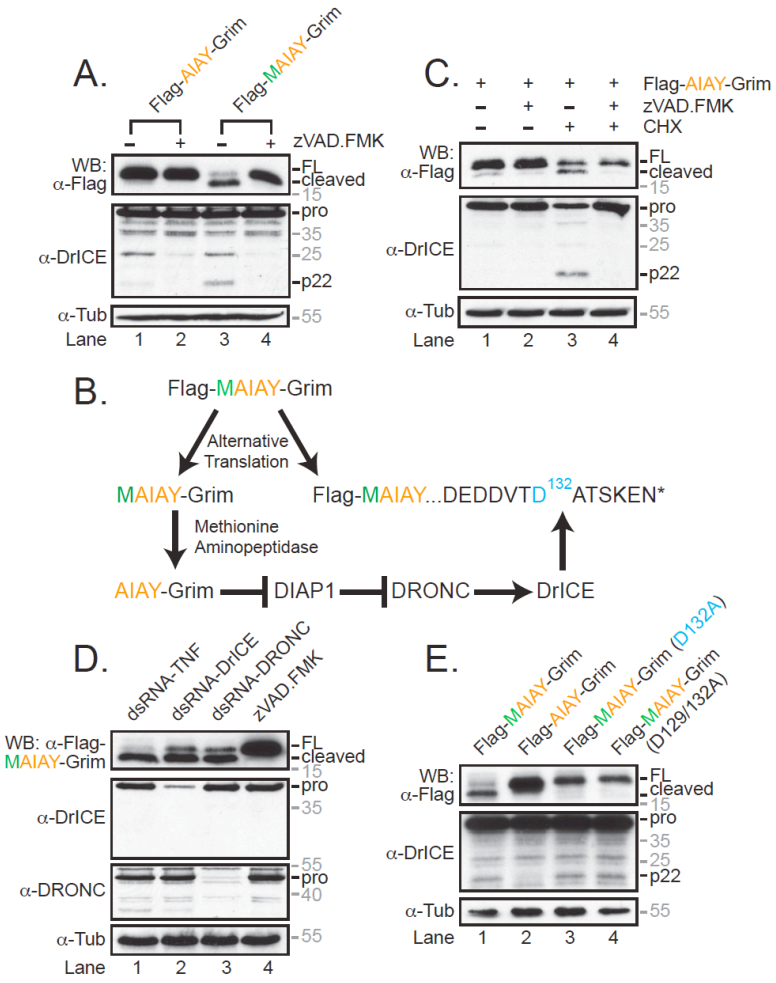

Figure 3.1 Grim is cleaved by caspases at C-terminus. (*A*) pIE-Flag-AIAY-Grim and pIE-Flag-MAIAY-Grim were expressed in S2 cells with or without the poly-caspase inhibitor, zVAD-fmk (50 μM). Cell lysates were then immunoblotted for Flag and DrICE. The AIAY sequence (orange) is the natural IBM in Grim and the Methionine (green) in Flag-M-AIAY served as an alternative translational start site when fused downstratem of the Flag tag. (*B*) Proposed scheme to explain the C-terminal processing of Flag-MAIAY-Grim by caspases at Asp132. (*C*) S2 cells were transfected with pIE-Flag-AIAY-Grim and treated with CHX (10 μM) with our without zVAD-fmk. In the absence of zVAD-fmk, CHX induces caspase activation and apoptosis. Following treatment, cell lysates were prepared and immunoblotted for Flag-tagged Grim or endogenous DrICE. (*D*) S2 cells were treated with dsRNA to human TNF (control), DrICE or DRONC for 24h, followed by transfection with pIE-Flag-MAIAY-Grim. As a positive control, cells were also treated with zVAD-fmk to inhibit caspases and prevent Grim cleavage. (*E*) pIE-Flag-MAIAY-Grim was mutated at Asp132 or Asp129/132 and transfected into S2 cells. pIE-Flag-AIAY-Grim was used as a positive control, as it does not undergo alternative translation and C-terminal cleavage. After 24h, cell lysates were prepared and immunoblotted for Flag-tagged Grim and endogenous DrICE.

3.3.2 DIAP1 ubiquitinates Grim at a single lysine residue that is removed by caspases.

As previously noted, IAP antagonists can be ubiquitinated by IAPs (Hao et al., 2004; Hu and Yang, 2003; MacFarlane et al., 2002; Olson et al., 2003b), and, while mapping the caspase cleavage site for Grim, we noted the presence of a single lysine, located downstream of the caspase cleavage site (Fig. 3.2*A*). This led us to question whether DIAP1 ubiquitinated Grim at Lys136 in cells and ultimately whether removal of its C-terminus could protect Grim from proteasomal-mediated degradation (Fig. 3.2*A*). Although the usefulness of our Flag-MAIAY-Grim construct was immeasurable in initially revealing Grim cleavage (Fig. 3.1), its processing was somewhat complicated in that Flag-MAIAY-Grim could not interact with DIAP1, and AIAY-Grim could not be detected with an anti-Flag antibody. Therefore, moving forward, we chose instead to use a Grim-HSV-His_6 construct, as it contained a fully functional IBM, capable of binding DIAP1 (as detailed later), and, unlike Flag, the HSV-His_6 tag did not contain a lysine residue to interfere with our ubiquitination studies. Of course, Grim cleavage prevented us from visualizing HSV tag with an anti-HSV antibody, but this could be easily overcome by mutating Asp132 to alanine.

We first examined whether Grim is ubiquitinated endogenously. We did ubiquitination assay in cells that only express Grim and Flag-ubiquitin in the presence of proteasome inhibitor MG132 and caspase inhibitor, zVAD-fmk. Even though Grim (D132A) was weakly ubiquitinated, the lysine mutant, Grim (D132A/K136R) [henceforth known as Grim (DA/KR)] was not modified at all (Fig. 3.2*B*). We also did the ubiquitination assay by pulling down His-tagged Grim using Ni^{2+}-NTA beads, and the

amount of Grim ubiquitination was little but still detectable (Fig. 3.2*D*, lane 1). We suspected it is due to the robust regulation of the copper promoter upstream of Grim constructs, so there were not enough E3 to ubiquitinate Grim. To overcome the lack of E3, we overexpressed proportional amount of DIAP1 and Grim in cells and found DIAP1 coexpression improved our detection of Grim-ubiquitin conjugates (Fig. 3.2*C* and Fig. 3.3*A*).

Notably, when we knocked down DIAP1 in cells, there is no obvious reduction in Grim ubiquitination (Fig. 3.2*D*, lanes 3 and 4), which is likely due to the functional redundancy of DIAP2 or other BIR domain containing E3. Indeed, when coexpressed with DIAP1 or DIAP2, Grim (D132A) underwent time-dependent ubiquitination that was not observed with Grim (DA/KR; Fig. 3.2*C* and 3.3*B*). Consistent with these data, in pulse-chase experiments, Grim (D132A) was rapidly degraded ($t1/2 < 2$ h) in cells, whereas Grim (DA/KR) was resistant to turnover ($t1/2 > 8$ h; Fig. 3.4*A* and *B*). Treatment with the proteasome inhibitor MG132 also resulted in the accumulation of Grim (D132A) and its ubiquitinated species in the presence and absence of ectopically expressed DIAP1 (Fig. 3.3*C*, lanes 1 and 3 and Fig. 3.3*D*, lanes 1 and 2). Loss of Grim was not caused simply by increased cell death, as ubiquitination and degradation of Grim (D132A) occurred even in the presence of zVAD-fmk (Fig. 3.3*D*, lanes 5 and 6, and Fig. 3.4*A*). Finally, because caspase cleavage generates a large N-terminal fragment of Grim, we examined the fate of Grim (1-131) and found that it persisted in cells and retained its ability to bind DIAP1 (Fig. 3.4*C*, and Fig. 3.5, lanes 2 and 3). Thus, DIAP1 mediates the turnover of Grim by ubiquitinating it on Lys136, but turnover can be suppressed by caspase cleavage at Asp132 without disrupting its interaction with DIAP1.

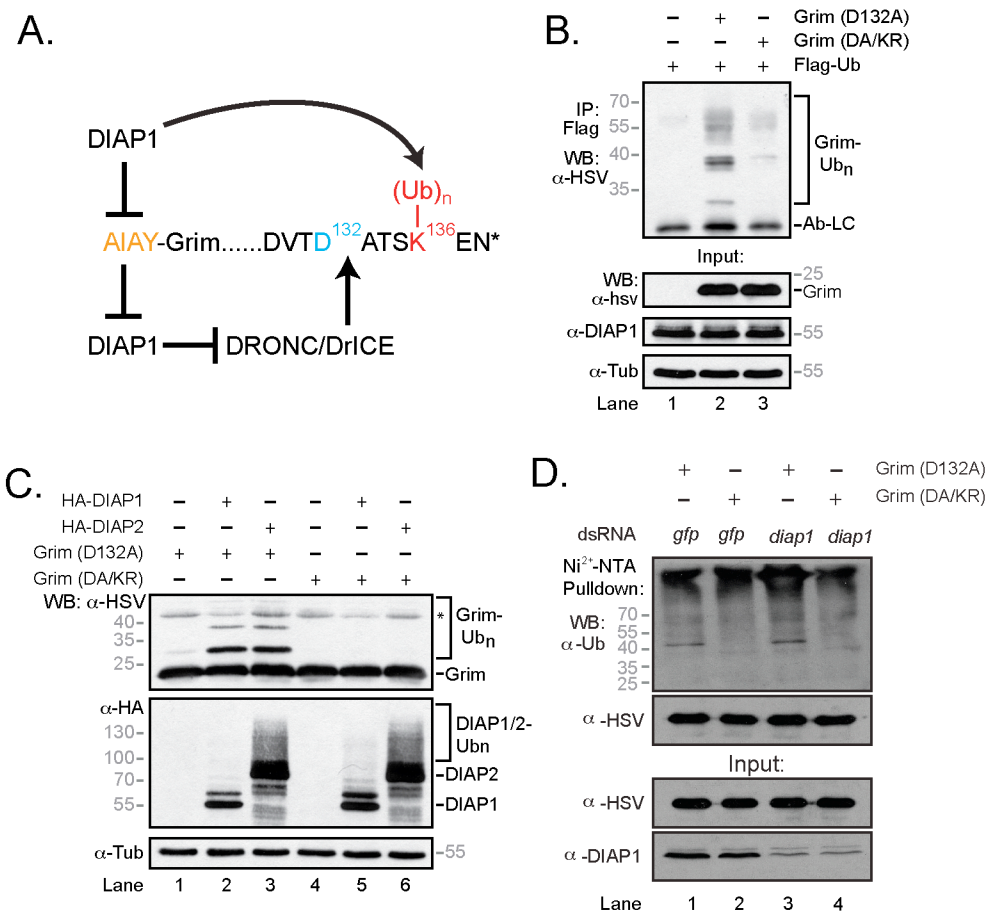

Figure 3.2 Grim is ubiquitinated at Lys136. (*A*) Proposed relationship between DIAP1-mediated ubiquitination of Grim at Lys136 (Red) and caspase cleavage of Grim at Asp132 (blue). (*B* and *D*) Grim ubiquitination with E3 ligases at endogenous levels. S2 cells were transfected with either the cleavage mutant pRmHa3-Grim (D132A)-HSV-His or the double mutant Grim (DA/KR)-HSV-His along with pMT-Flag-ubiquitin (*B*) or dsRNA (*D*). Grim-ubiquitin conjugates were isolated by an anti-Flag antibody (*B*) or Ni^{2+}-NTA beads (*D*). The precipitates were immunoblotted for indicated antibodies. (*C*) S2 cells were cotransfected with pIE-HA-DIAP1 or HA-DIAP2 and pRmHa3-Grim (D132A)-HSV or Grim (DA/KR)-HSV. After 24h, cells were incubated with $CuSO_4$ (0.7 mM) for an additional 8 h to induce Grim expression, and lysates were prepared and immunoblotted for HSV and HA.

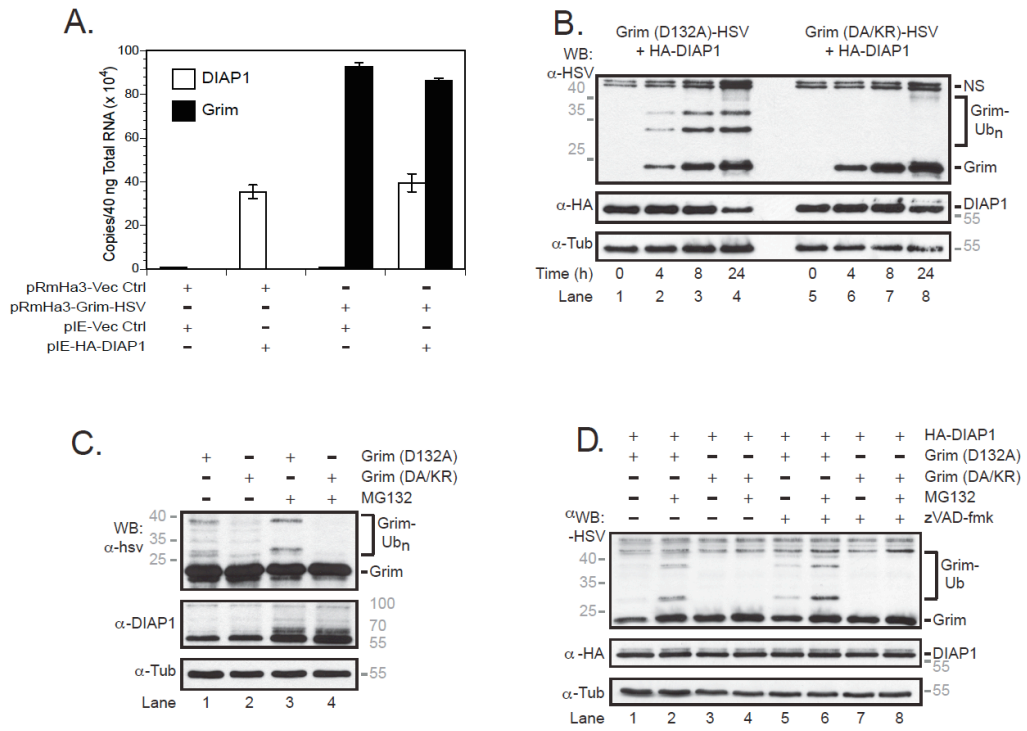

Figure 3.3 Grim is ubiquitinated by DIAP1 at Lys136 in a time-dependent manner. (*A*) Proportional expression of DIAP1 and Grim in S2 cells. Cells were transfected with HA-DIAP1 and/or Grim-HSV-His$_6$ and total RNA was isolated for quantitative PCR analysis. The Ct values were applied to standard curves generated for *diap1* and *grim*. Results are presented was the number of mRNA copies present in 40ng of total RNA. (*B*) S2 cells were cotransfected with pIE-HADIAP1 and either the cleavage mutant pRmHa3-Grim (D132A)-HSV-His or the double mutant Grim (DA/KR)-HSV-His. After 24 h, Grim expression was induced by $CuSO_4$, and samples were collected at indicated time points for Western blot analysis. (*C* and *D*) S2 cells were transfected with either Grim(D132A) or Grim (DA/KR) alone (*C*) or along with DIAP1 (*D*). After transfection, cells were treated with DMSO, MG132, or/and zVAD-fmk for 8 h. Lysates were analyzed by Western blotting.

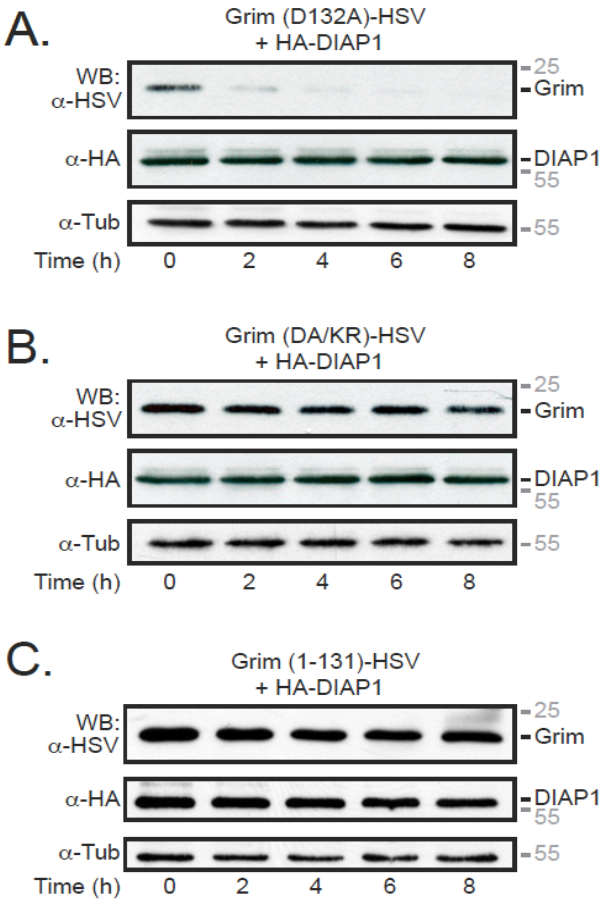

Figure 3.4 The half-life of Grim and its mutants. S2 cells were transfected with Grim (D132A), Grim (DA/KR), or Grim (1-131) along with pIE-HA-DIAP1. Cells were then induced with $CuSO_4$ for 2h, washed with PBS solution, and incubated in normal medium containing zVAD-fmk and CHX (10 μg/ml). At various time points (0-8 h), lysates were prepared an analyzed with immunoblotting.

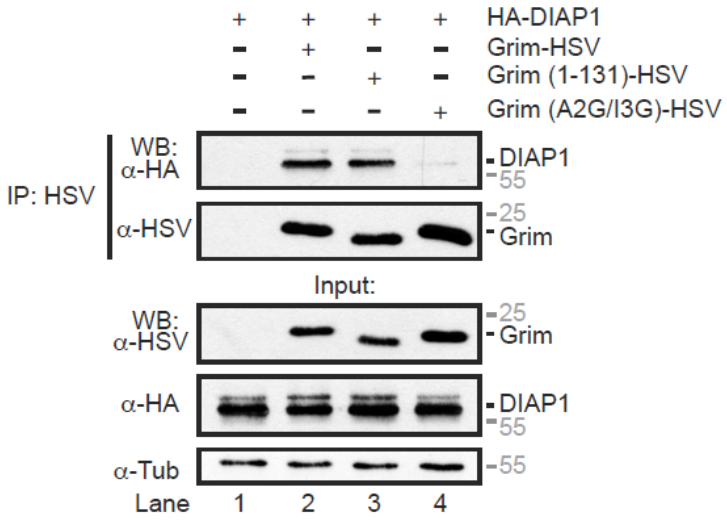

Figure 3.5 Co-immunoprecipitation of Grim-HSV and HA-DIAP1. S2 cells were transfected with Grim, Grim (1-131), or the IBM mutant Grim (A2G/I3G), which is unable to interact with DIAP1. Grim and Grim mutants were then immunoprecipitated by an anti-HSV antibody, and the precipitates were immunoblotted for HA-DIAP1.

3.3.3 Grim self-associates and is ubiquitinated by DIAP1 in a BIR2 and RING-dependent manner.

To further characterize the mechanisms of Grim ubiquitination, we next examined several DIAP1 mutants and did so in the presence of zVAD-fmk to prevent cell death without interfering with Grim ubiquitination (Fig. 3.3*D*, lanes 2 and 6). As expected, removal of the RING domain from DIAP1—or introduction of a RING mutation into full-length DIAP1—prevented Grim ubiquitination entirely (Fig. 3.6*A*, lanes 5 and 6, and Fig. 3.6*B*, lanes 3 and 4). Surprisingly, however, a BIR2-RING fragment was approximately equal to full-length DIAP1 at ubiquitinating Grim (Fig. 3.6*A*, compare lanes 3, 4, 7, and 8), and a BIR2 mutation (H283Y) in full-length DIAP1 or the BIR2-RING fragment diminished or abolished Grim ubiquitination altogether (Fig. 3.6*B*, lanes 7 and 8, and Fig. 3.6*C*, lanes 3 and 4). A BIR1 mutation (W90A), on the contrary, had no significant impact on Grim ubiquitination (Fig. 3.6*B*, compare lanes 1, 2, 5, and 6), but, when combined with the BIR2 mutation, DIAP1 (W90A/H283Y) was largely defective in its ability to bind and ubiquitinate Grim (Fig. 3.7*A*, lanes 2 and 7, and Fig. 3.6*C*, lanes 7 and 8).

The fact that a BIR2 mutant negatively impacted Grim ubiquitination more than a BIR1 mutant, even though Grim binds to BIR1 with higher affinity (Wu et al., 2001; Yan et al., 2004), suggested that the proximity of Grim to the RING domain affected Grim ubiquitination more so than differences in binding affinity. To test this hypothesis, we generated a ΔBIR2 fragment and found that this internal BIR2 deletion mutant ubiquitinated Grim almost as efficiently as the BIR2-RING fragment (Fig. 3.6*C*, lanes 1, 2, 5, and 6). Interestingly, we also noted that incorporation of the H283Y mutation into

full-length DIAP1 dramatically enhanced DIAP1 autoubiquitination, regardless of the BIR1 mutation (Fig. 3.6*B*, lanes 7 and 8, and Fig. 3.6*C*, lanes 7 and 8), but had little effect on the BIR2-RING fragment, which failed to undergo significant autoubiquitination (Fig. 3.6*C*, lanes 3 and 4). Thus, DIAP1 preferentially ubiquitinates BIR2-bound Grim, but in the absence of a BIR2-bound substrate, DIAP1 appears to ubiquitinate itself somewhere in or around the BIR1 domain (Fig. 3.7*C*).

Finally, we noted that, whereas ΔBIR2 (W90A) and BIR2-RING (H283Y) were fully defective in Grim binding, full-length DIAP1 (W90A/H283Y) retained a weakened but persistent interaction with Grim (Fig. 3.7*A*, lanes 2, 4, 6, and 7). Recent studies suggest that some IAPs and IAP antagonists can homo- and heterodimerize (Chai et al., 2000; Dueber et al., 2011; Feltham et al., 2011; Sandu et al., 2010; Silke et al., 2005), leading us to speculate that a Grim dimer might form a more stable interaction with DIAP1 (W90A/H283Y) by forming simultaneous low-affinity interactions with BIR1 and BIR2. To determine if Grim could self-associate in cells, we coexpressed and immunoprecipitated differentially tagged IBM mutants of Grim [i.e., Grim (A2G/I3G)-HSV and Grim (A2G/I3G)-myc] and discovered that indeed Grim could self-associate (Fig. 3.7*B*, lane 3), independently of its interaction with DIAP1 (Fig. 3.5, compare lanes 2 and 4). Thus, we propose that Grim dimerizes and engages DIAP1 through interactions with its BIR1 and BIR2 domains, similar to the manner in which dimerized Smac interacts with XIAP, but only BIR2-bound Grim undergoes significant RING-dependent ubiquitination (Fig. 3.7*C*).

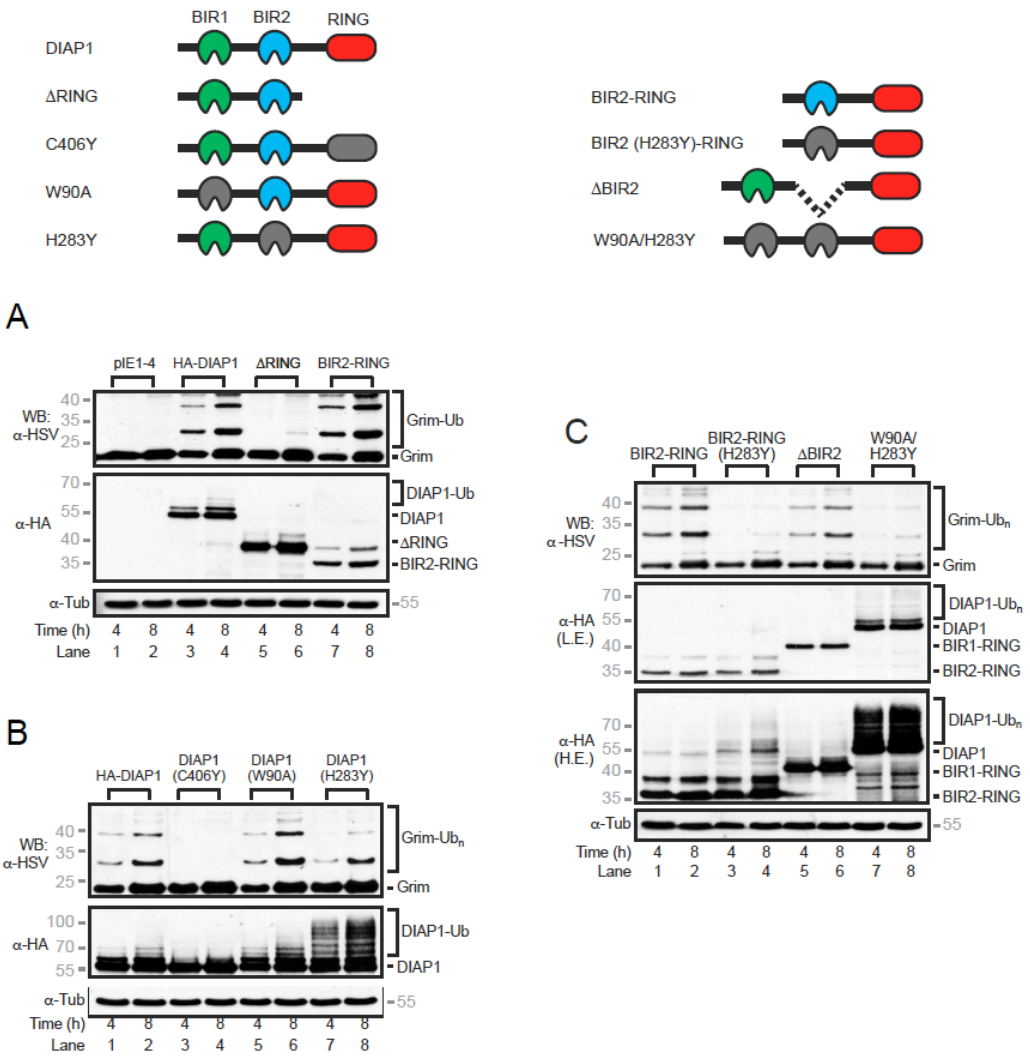

Figure 3.6 DIAP1-BIR2 preferentially ubiquitinates Grim in a RING-dependent manner. DIAP1 truncation and point mutants; BIR1 (green), BIR2 (dark bule), or RING (red) domains were deleted or mutated (gray) as indicated. (*A-C*) S2 cells were transfected with pRmHa3-Grim-HSV along with indicated DIAP1 construct. Cell lysates were collected at 4 and 8 h post induction and analyzed by Western blotting.

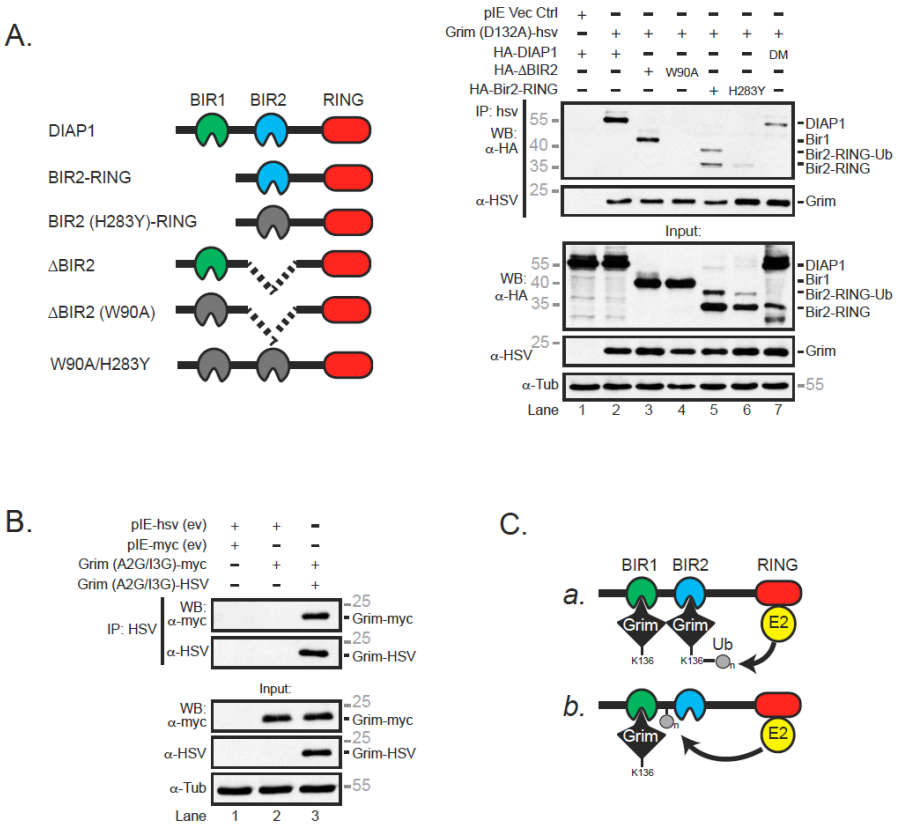

Figure 3.7 DIAP1-BIR2 preferentially ubiquitinates dimerized Grim in a RING-dependent manner. DIAP1 truncation and point mutants; BIR1 (green), BIR2 (dark blue), or RING (red) domains were deleted or mutated (gray) as indicated. (*A*) S2 cells were transfected with pRmHa3-Grim-HSV along with indicated DIAP1 construct. Afterward, immunoprecipitations were performed by using an anti-HSV antibody, and Grim complexes were analyzed with immunoblotting. Input control blots for each protein were also performed (lower). (*B*) S2 cells were cotransfected with Grim IBM mutants, pRmHa3-Grim (A2G/I3G)-HSV and/or pRmHa3-Grim (A2G/I3G)-myc. Afterwards, immunoprecipitations were performed by using an anti-HSV antibody, and Grim (A2G/I3G) complexes were analyzed by Western blotting. (*C*) Cartoon illustrates that (*a*) DIAP1 preferentially ubiquitinates BIR2-bound Grim in a RING-dependent manner, (*b*) in the absence of BIR2 binding (as a result of a loss-of-function mutation), DIAP1 ubiquitinates itself.

3.3.4 Grim is polyubiquitinated by DIAP1 and the E2 ubiquitin-conjugating enzyme UbcD1, primarily through K48-linked chains

Previous studies have suggested that Reaper and Grim-induced autoubiquitination of DIAP1 is mediated by the E2 ubiquitin-conjugating enzymes, Morgue and UbcD1, and that Grim catalyzes this process through direct binding to UbcD1 (Hays et al., 2002; Ryoo et al., 2002; Wing et al., 2002; Yoo, 2005). Therefore, to determine the roles of Morgue and UbcD1 in DIAP1-mediated ubiquitination of Grim, we performed knock down experiments in S2 cells and found that depletion of UbcD1, but not Morgue, significantly reduced polyubiquitination of Grim (Fig. 3.8*A*). Because DIAP1 can catalyze both K48 and K63 ubiquitin linkages (Ditzel et al., 2008), we next sought to establish the type of linkages present in ubiquitinated Grim. S2 cells were transfected with DIAP1, along with either Grim (D132A) or Grim (DA/KR), and, after 24 h, Grim proteins were isolated and immunoblotted with antibodies specific for endogenous K48 or K63-linked ubiquitin chains. Importantly, Grim (D132A), but not Grim (DA/KR), underwent DIAP1-dependent K48-linked ubiquitination (Fig. 3.8*B*, lanes 2 and 4), consistent with the observed turnover of Grim (Fig. 3.4) and the accumulation of Grim-ubiquitin conjugates following treatment with MG132 (Fig. 3.3*D*, lanes 1, 2, 5, and 6).

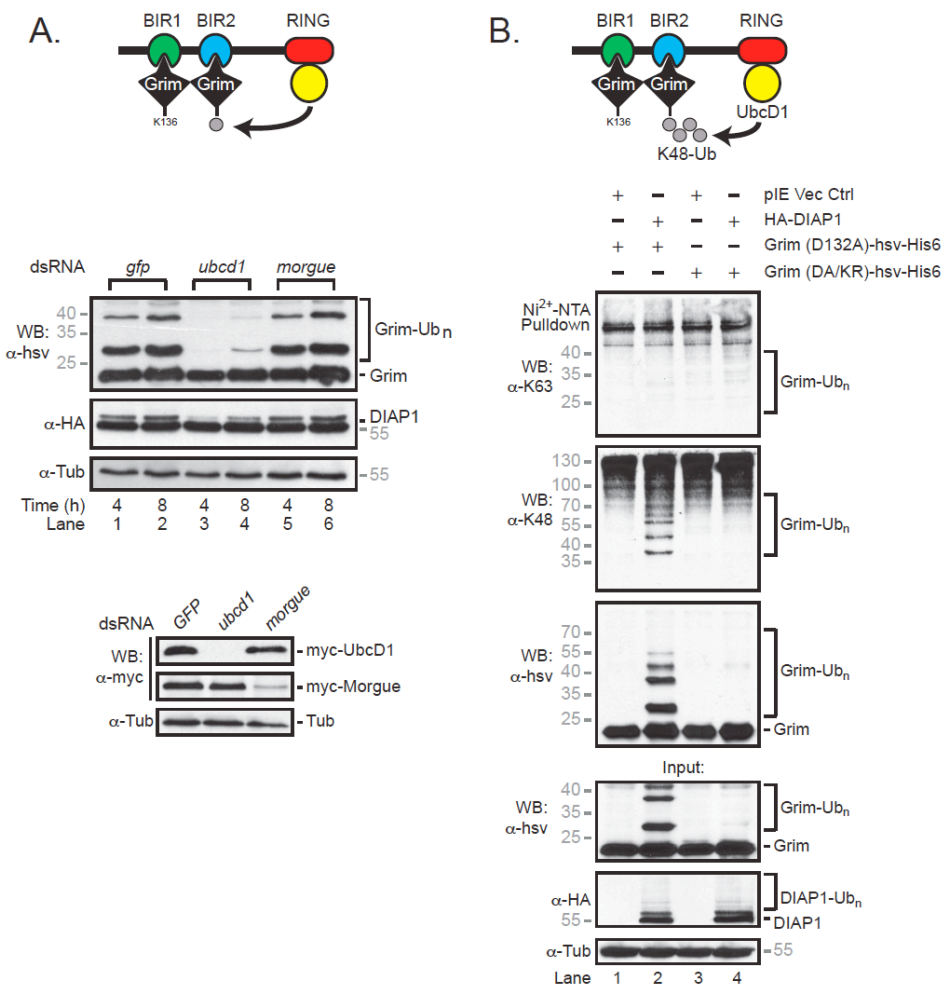

Figure 3.8 DIAP1 catalyzes UbcD1-dependent K48 based ubiquitination of Grim. (*A*) S2 cells were transfected with dsRNA to *gfp* (control), *ubcd1*, or *morgue* for 24 h, after which the cells were cotransfected with Grim-HSV and HA-DIAP1 for an additional 24 h. Alternatively, cells were cotransfected with myc-UbcD1 or myc-Morgue expression constructs to determine the effectiveness of the RNAi (left). Lysates were prepared and immunoblotted with indicated antibodies. (*B*) S2 cells were cotransfected with HA-DIAP1 and Grim (D132A)-HSV-His_6 or the DA/KR mutant. Lysates were prepared under denaturing conditions, and Grim was precipitated by using Ni^{2+}-NTA beads and immunoblotted with antibodies specific for K48- or K63-based ubiquitin linkages, as well as the C-terminal HSV tag.

3.3.5 DrICE removes the C-terminus from Grim-ubiquitin conjugates and inhibits Grim turnover.

K48-linked ubiquitin chains of four or more were once thought to be essential for proteasome-mediated degradation of a substrate (Thrower et al., 2000). A more recent study, however, indicates that chain length requirements depend upon the size of the substrate, with smaller substrates requiring shorter chains (Shabek et al., 2012). In our experiments, we primarily observed Grim-ubiquitin chains of two to four ubiquitins in length (Grim-ubiquitin2–4). This was not a result of interference of the ubiquitin chains with the C-terminal tag, as extending the C-terminus (3×HSV) failed to significantly improve our capture of higher-order Grim-ubiquitinn > 5 conjugates (Fig. 3.9). Thus, the addition of four or fewer ubiquitins to Grim appears to be sufficient to mediate its turnover. We similarly questioned whether polyubiquitination of Grim at Lys136 might interfere with its caspase cleavage at Asp132. Therefore, we expressed Grim in S2 cells in the presence of zVAD-fmk, washed the cells, prepared lysates, and incubated them with or without recombinant DrICE in vitro. As expected, Grim (D132A) was ubiquitinated, but neither the conjugated nor unconjugated forms were susceptible to DrICE cleavage (Fig. 3.10, lanes 5 and 6). Grim, Grim-ubiquitin conjugates, and Grim (K136R), on the contrary, underwent robust cleavage by DrICE (Fig. 3.10, lanes 2 and 4). Moreover, by using a recently available polyclonal antibody to Grim, we observed the appearance of a single N-terminal fragment of Grim (1-132), indicating that Grim and its ubiquitinated species were cleaved by DrICE (Fig. 3.10, Middle panel, lanes 2 and 4). Thus, DrICE can prevent proteasomal-mediated turnover of Grim and extend its half-life in cells by removing its C-terminus before or after ubiquitination.

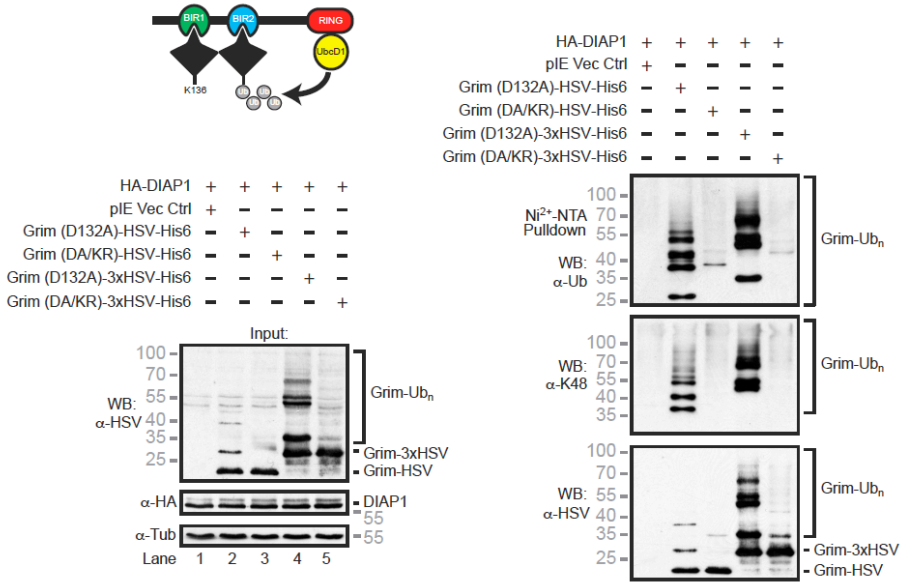

Figure 3.9 Grim-ubiquitin chains are composed primarily of four or fewer ubiquitins. S2 cells were cotransfected with HA-DIAP1 and Grim (D132A)-HSV-His_6, Grim (D132A)-3xHSV-His_6, Grim (DA/KR)-HSV-His_6, or Grim (DA/KR)-3xHSV-His_6. Lysates were prepared under denaturing conditions, and Grim was precipitated by using Ni^{2+}-NTA beads and immunoblotted with antibodies specific for ubiquitin, K48-based ubiquitin linkages, and the C-terminal HSV tag.

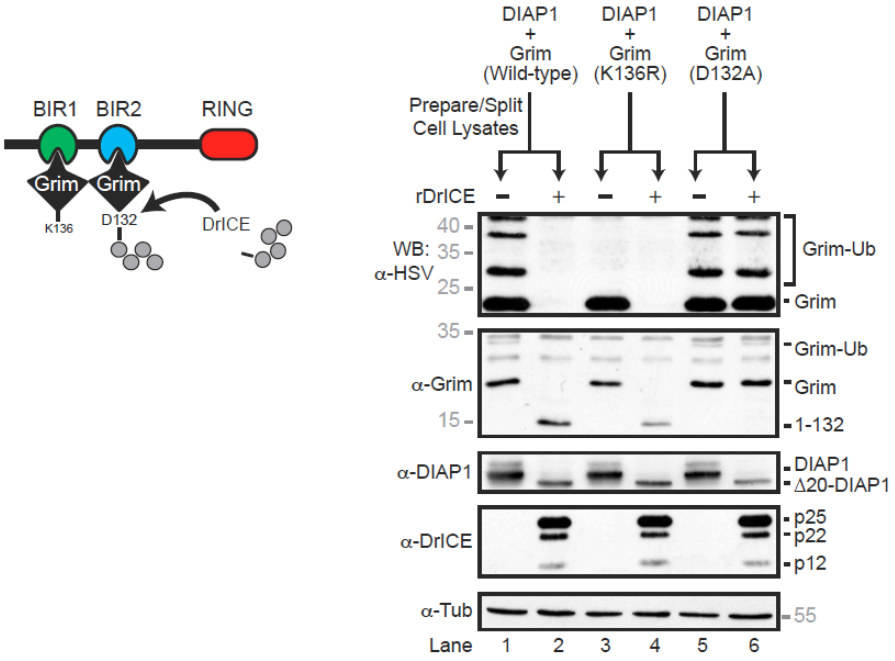

Figure 3.10 DrICE can remove Grim's C-terminus and covalently linked ubiquitin chains. S2 cells were cotransfected with HA-DIAP1 and WT Grim, Grim (D132A), or Grim (K136R). After 24h, cells were incubated with $CuSO_4$ (0.7 mM) for an additional 8 h in the presence of zVAD-fmk (50 μM) and MG132 (10 μM) to prevent caspase activity and proteasomal-dependent turnover of proteins. After washing the cells, lysates were prepared and incubated with or without recombinant DrICE for 30 min at 37ºC, followed by SDS/PAGE/immunoblotting.

3.3.6 Grim cleavage and ubiquitination impact the kinetics of caspase activation and cell Death.

Finally, to determine the impact of caspase cleavage on Grim-induced caspase activation and cell death, we expressed Grim in S2 cells, measured DEVDase activity at 6 h, and used time-lapse DIC and fluorescence microscopy to quantify apoptosis. As expected, Grim (D132A) produced less caspase activity and cell death over time, compared with WT Grim, in agreement with the fact that noncleavable Grim is ubiquitinated and degraded regardless of caspase activity (Fig. 3.11). Conversely, Grim (K136R), Grim (DA/KR), and the N-terminal cleavage fragment Grim (1-131) could not be ubiquitinated and therefore supported greater levels of caspase activation and cell death, particularly compared with noncleavable Grim (Fig. 3.11). Thus, cleavage of Grim sustained its presence in the cell and facilitated a feed-forward caspase amplification loop, wherein cleaved Grim was allowed to continue its antagonism of DIAP1, thereby enhancing caspase activation and cell death.

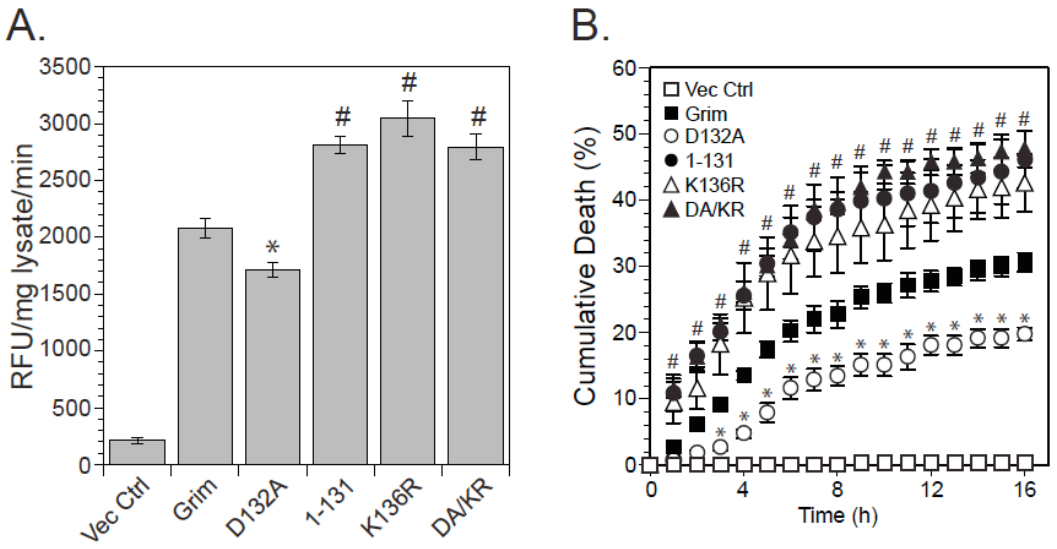

Figure 3.11 Grim cleavage and lysine mutants exhibit opposite effects on caspase activation and cell death. S2 cells were cotransfected with GFP-DIAP1 and Grim or one of its mutants. (*A*) Lysates were then prepared at 6h and assayed for effector caspase DEVDase activity, or (*B*) cells were monitored by using time-lapse DIC and fluorescence microscopy to determine the cumulative amount of cell death over time, as determined by cellular blebbing and formation of apoptotic bodies. [$^{*}P < 0.05$, Grim (D132A) is significantly different from WT Grim; $^{\#}P < 0.05$, Grim (K136R), Grim (DA/KR), and Grim (1-131) are significantly different from Grim (D132A) and WT Grim.]

3.4 Discussion

Caspases play an undeniable role in the progression of apoptosis, mediating the cleavage of numerous substrates (>800 in mammals) and the eventual dismantling of the cell (Luthi and Martin, 2007). For certain substrates removal of a regulatory domain results in their constitutive activation, as in the case of various kinases (Emoto et al., 1995; Graves et al., 1998). For others, cleavage results in a loss of function, and the resulting fragments are frequently degraded by the 26S proteasome (Demontis et al., 2006; Landais et al., 2006; Plesca et al., 2008; Sears et al., 2011; Semple et al., 2007; Tikhomirov and Carpenter, 2001). In this study, we found an interaction between caspases and the ubiquitin-proteasome system. Caspase cleavage of the IAP antagonist Grim, at its C-terminus, removes a critical lysine before or following its ubiquitination by DIAP1. In doing so, caspases sustain the presence of Grim in the cell and allow it to further activate caspases by antagonizing additional DIAP1 (Fig. 5.1). Thus, in effect, caspases compete with the proteasome to determine the fate of Grim and, by extension, the fate of the cell.

Most of the previous studies on Grim have focused on its acute cell death effects, with little consideration as to how this IAP antagonist is regulated at the posttranscriptional level. Grim plays a selective role in the death of glial cells in the microchaete of flies and in regulating the number of neuroblasts during development of the ventral nerve cord (Tan et al., 2011; Wu et al., 2010). Loss of Grim did not cause dramatic developmental defects, but Grim is the only IAP antagonist that induces embryo midline cell death when ectopically expressed. It is possible that Grim levels are constantly suppressed transcriptionally or post-translationally so that Grim is only

available for specific or limited cells to eliminate themselves during development or metamorphosis. It's worth noting that the C-terminal sequence of Grim is conserved among almost all the sequenced *Drosophila* species (Fig. 3.12), so this regulation between caspase activity and the proteosomal system might be important regulation for proper caspase activity at a specific developmental timeframe. There are examples in which spatiotemporal control of caspase activity is required to induce differentiation while preventing cell death. For example, caspases play important roles in spermatid individualization (Arama et al., 2003; Huh et al., 2003), and Grim is expressed in Malpighian tubules during metamorphosis, implying that it plays a role in the formation of these tubules, but its expression is largely restricted to the nucleus (Shukla and Tapadia, 2011; Tapadia and Gautam, 2011). Thus, the interplay among DIAP1, caspases, Grim, and the proteasome may constitute a unique form of rheostatic control, such that, at relatively low levels of Grim, DIAP1 may allow for some caspase activity while limiting through ubiquitination the overall levels of Grim and active caspases. In this model, as Grim expression reaches a threshold, the level of caspase activity becomes sufficient to liberate Grim's C-terminus, thereby preserving its presence in the cell and further enhancing caspase activation.

The wider prevalence of an interaction between caspases and the ubiquitin-proteasome system will require further investigation in the future. However, a cursory examination of the UniProt/Swiss-Prot database indicates that 197 proteins (including splice variants) contain a single lysine in their coding sequences, and a number of these proteins contain a predicted caspase cleavage site(s) that, if genuine, would liberate the lysine from the N-or C-terminus of the protein. Moreover, proteins with multiple lysines

need not be excluded from this list, as there are examples in which a particular lysine still represents the primary target for ubiquitination (Bornstein et al., 2012). Although caspase cleavage of Grim enhances cell death, the preservation of other caspase substrates, such as transcription factors, could mediate nonapoptotic events, including differentiation. Finally, this type of regulation may not be restricted to caspases, as proteolytic removal of a key lysine could be performed by other proteases, including calpains and cathepsins, revealing novel interactions between these proteases and the ubiquitin-proteasome system.

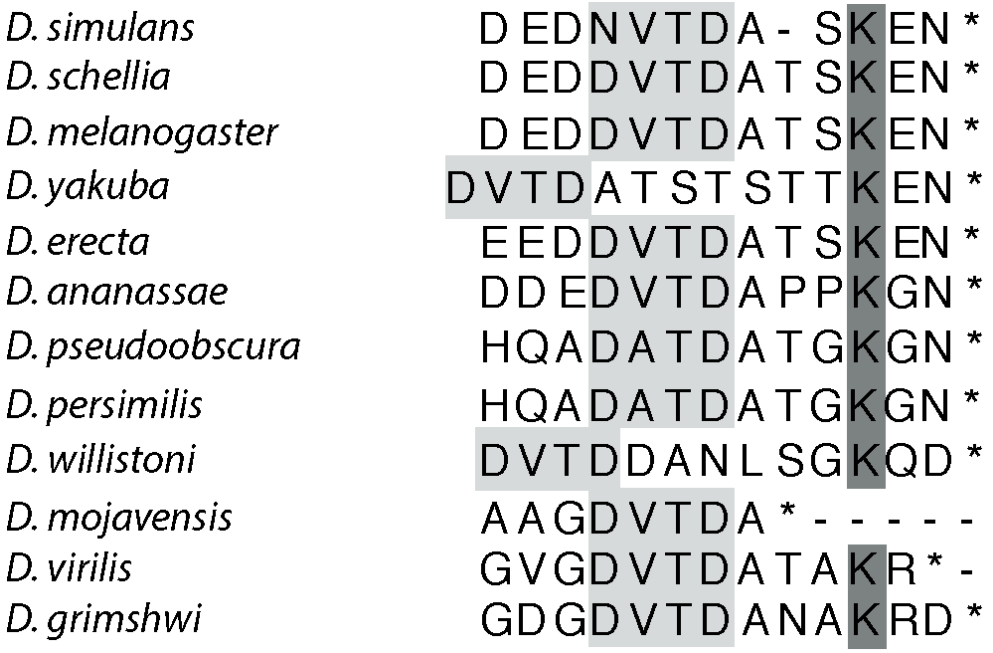

Figure 3.12 The C-terminal domain of Grim among different *Drosophila* species. The caspase cleavage site was highlighted as gray color and the lysine residue was labeled in dark gray. Almost all the sequenced species (except for *D. mojavensis*) have the conserved regulation of Grim levels by utilizing caspases and the proteasome system.

CHAPTER4

Characterization of Grim-induced cell death through an unconventional mechanism

4.1 Summary

Drosophila inhibitor of apoptosis 1 (DIAP1) prevents cell death in flies by interacting with and ubiquitinating caspases, leading to their inhibition or degradation. As an endogenous inhibitor of DIAP1, Grim utilizes its N-terminal IAP binding motif (IBM) to promote cell death through displacement of caspases and by inducing autoubiquitination and degradation of DIAP1. When cells are under stress, Grim expression is upregulated to liberate caspases from DIAP1 inhibition, leading to cell death. Herein, we demonstrate that in addition to its IBM, Grim can induce cell death through an IBM-independent pathway involving its Grim Helix 3 (GH3) domain and its C-terminus. Based on deletion analysis, both the GH3 and C-terminus are required and either region alone is insufficient to cause cell death. By using fluorescence-tagged proteins, we have determined that amino acids 110-124 are important for directing Grim to processing (P)-bodies (or DCP1-containing granules), where untranslated mRNA accumulates. Since the GH3 or C-terminal domains alone do not induce death, it appears that Grim needs to localize to the right spot in the cell to induce IBM-independent cell death. Loss of a P-body-localized translational repressor, Me31B, reduced Grim-induced cell death, suggesting that IBM-independent cell death occurs at least in part, through Me31B-mediated translational repression. In summary, Grim localizes to P-bodies

through amino acids 110-124, where it forms a complex with DCP1 and Me31B to induce IBM-independent cell death.

4.2 Introduction

Apoptosis is usually catalyzed by a series of evolutionarily-conserved pathways, involving the activation of caspases in order to execute cell death. Caspase activities can be suppressed by IAPs through direct IAP binding or sometimes through its E3-ligase-activity. IAPs are regulated by their endogenous inhibitors, IAP antagonists, which contain IBM domains that are capable of interacting with IAPs and promoting their inactivation or autoubiquitination (Dueber et al., 2011; Silke et al., 2004; Yoo, 2005). In flies, DIAP1 prevents cell death through caspase inhibition, but it can be compromised by IAP antagonists such as Grim, Reaper, or Hid. Some IAP antagonists, however, are bi-functional and possess proapoptotic activity outside of their IBMs (Chen et al., 2004; Claveria et al., 2002; Olson et al., 2003a). For example, dOmi degrades DIAP1 via its serine protease activity in an IBM-independent manner following its release from the mitochondrial intermembrane space (Challa et al., 2007). IBM-less versions of Reaper induce cell death by several proposed mechanisms: direct or indirect targeting of mitochondria resulting in GH3-dependent mitochondrial rupture (Freel et al., 2008; Olson et al., 2003a; Sandu et al., 2010), or alternatively, general translational repression resulting in downregulation of DIAP1 (Colon-Ramos et al., 2006; Holley et al., 2002; Tait et al., 2004). A third as of yet undefined mechanism involves neither mitochondria nor translational repression (Chen et al., 2004). Similarly, Grim can also induce apoptosis through an unconventional pathway, but the mechanism remains unclear. Grim can induce general translational repression *in vitro*, which may result in the

downregulation of short lived proteins, such as DIAP1 (Yoo et al., 2002), but whether Grim inhibits translation *in vivo* remains unclear. The GH3 region of Grim might contribute to some of its proapoptotic activity through mitochondrial targeting (Claveria et al., 2002), but the majority of Grim seems not to colocalize with mitochondria (Chen et al., 2004).

In the present study, we have discovered that instead of localizing to mitochondria, Grim localizes to the mRNP granule, P-bodes, where untranslated mRNA and proteins that regulate mRNA stabilities (such as DCP1 and Me31B) accumulate. Removing the C-terminus of Grim not only disrupts Grim localization to P-bodies (DCP1-containing granules), it impairs Grim-induced cell death. Strikingly, knockdown of a P-body-localized translational modulator, Me31B, reduced Grim-induced cell death, indicating that IBM-independent cell death requires Grim to associate with P-bodies or its components. Overall, we demonstrate that Grim localizes to P-bodies through its C-terminus, and it forms a complex with DCP1 and Me31B to induce IBM-independent cell death.

4.3 Results

4.3.1 Grim and its IBM mutant cause loss of GFP

In order to access the effect of IBM-independent activity at cellular levels, we set up the cell death assay in *Drosophila* Schneider's line 2 (S2) cells, a commonly used Drosophila embryonic cell line. The traditional Annexin V staining for examining apoptotic cells in mammals (Vermes et al., 1995) is not ideal for S2 cells due to a dramatic increase in cell fragmentation. Additionally, S2 cells do not have a clear Annexin V-positive population, which makes staining difficult. The cell death assay in *Drosophila* cells is usually performed by coexpression of a reporter construct (GFP or beta-galactosidase) along with the construct with genes of interest (Claveria et al., 2002; Olson et al., 2003a). The "cell death percentage" is calculated by measuring the loss of the reporter. To reduce the amount of singularly transfected cells, we used a 1:8 ratio of reporter to gene of interest. This ensures cells that express the reporter construct also express the gene of interest. We used GFP as our reporter and utilized flow cytometry to quantify GFP positive cells (Olson et al., 2003a). Since upregulation of Grim causes cell death, we cloned Grim downstream of the inducible metallothionein promoter, which can be stimulated by the addition of $CuSO_4$. Fig. 4.1*A* shows that wild-type Grim kills cells efficiently by 6 h after induction with copper. Similarly, the IBM mutant (GG-Grim), in which the first two amino acids in the IBM are replaced as Gly-Gly, also kills S2 cells but with slower kinetics. At each time point, cell survival was calculated as the relative percentage of GFP positive cells following copper induction compared to the uninduced sample. The pan-caspase inhibitor zVAD-fmk treatment prevented loss of GFP, indicating that caspases are responsible for Grim-induced cell death (Fig. 4.1*A*). When

effector caspase activity was measured by an effector caspase substrate, DEVD-AMC, we found that both Grim and GG-Grim stimulated DEVDase activity (Fig. 4.1*B*). DEVDase activities were higher at 6 h than 24 h, most likely because cells had begun to disintegrate at the later time point (Fig. 4.1*B*). Overall, our data demonstrate that Grim is a bi-functional killer and that while its N-terminal IBM enhance the speed of killing, the remainder is sufficient to kill through a slower and as yet uncharacterized mechanism. Moreover, the fact that the zVAD-fmk prevents both the IBM-dependent and –independent loss of GFP (Fig. 4.1*A*) indicates that caspase activities are also involved in IBM-independent cell death.

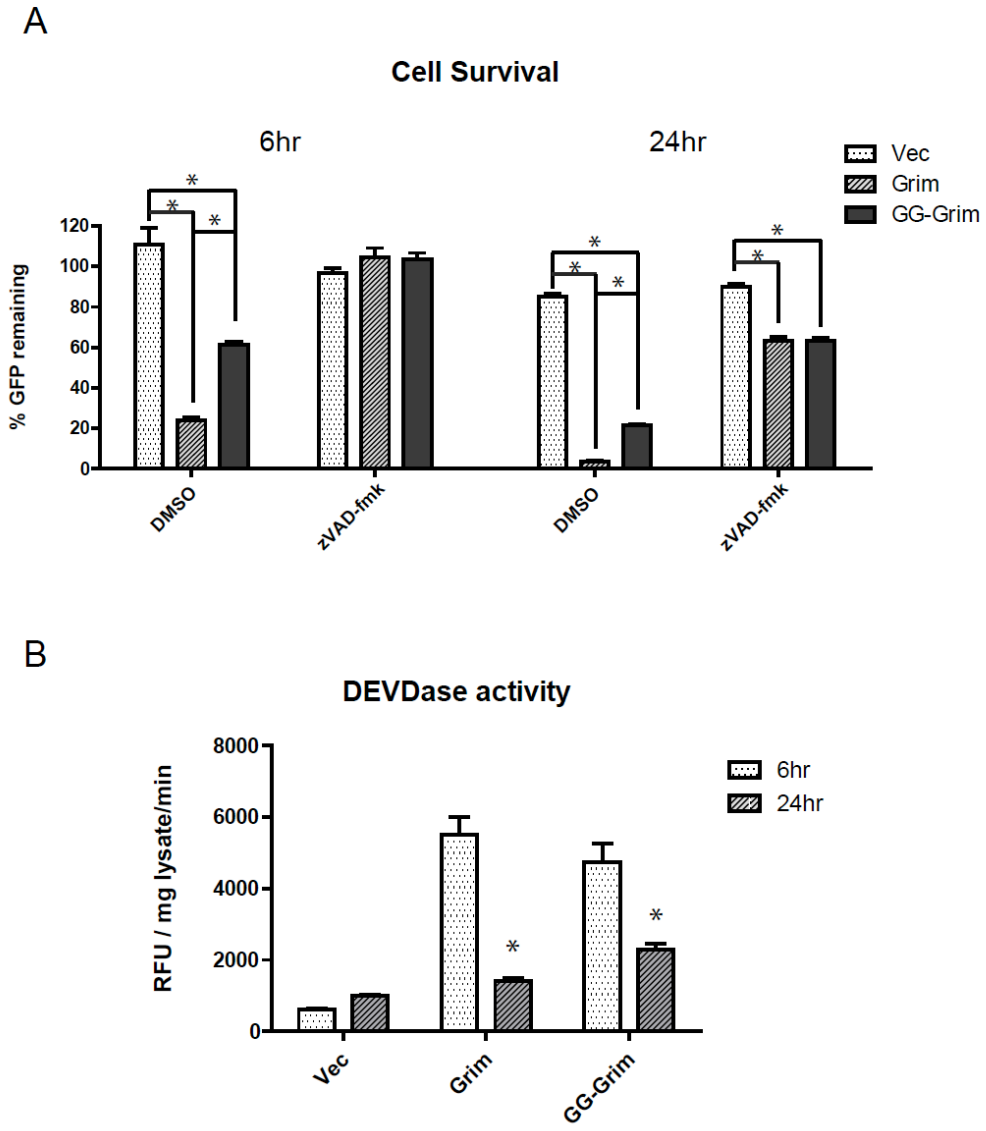

Fig. 4.1 Both Grim and GG-Grim induced zVAD-inhibitable cell death. S2 cells were transfected with 800 ng pRmHa3 plasmid DNA, pRmHa3-Grim-HSV, or GG-Grim-HSV along with 100 ng pAc-GFP. (*A*) After 16 h, cells were split and exposed to DMSO or zVAD-fmk in the presence or absence of $CuSO_4$. GFP positive cells were counted after 6 h or 24 h induction by flow cytometry. The percent of GFP positive cells was used to represent cell survival rate, and was calculated by 100% x (GFP^+ cells in the copper-treated sample) / (GFP^+ cells in the non copper-treated sample). The data were collected from four individual experiments. [$*P < 0.05$, the two groups indicated are significantly different.] (*B*) DEVDase activity assays were performed at 6 h or 24 h post-induction. Activity was calculated as the relative fluorescence units (RFU)/mg lysate/min. The data were collected from three individual experiments. [$*P < 0.05$, Grim or GG-Grim is significantly different from vector.]

4.3.2 The C-terminus of Grim is critical for its activity

To investigate the mechanism of Grim-IBM-independent cell death, we examined various Grim deletion mutants. When comparing GG-Grim with Grim Δ78, GG-Grim is a better killer at 6 h, suggesting that polyglutamine (polyQ) region might enhance killing at shorter time point (Fig. 4.2). Grim Δ78, however, kills as well as GG-Grim at 24 h, whereas Grim (79-105; GH3 region) or Grim (106-138; CTD) failed to kill (Fig. 4.2). When comparing GG-Grim with GG-Grim (1-105), or Grim Δ78 with Grim (79-105), it is obvious that the presence of the C-terminus is required for cell killing at least in the absence of Grim's IBM (Fig. 4.2). The IBM-independent cell death has always been correlated with Grim's localization (Claveria et al., 2002; Olson et al., 2003a); we therefore decided to investigate the localization of Grim, specifically the C-terminal domain.

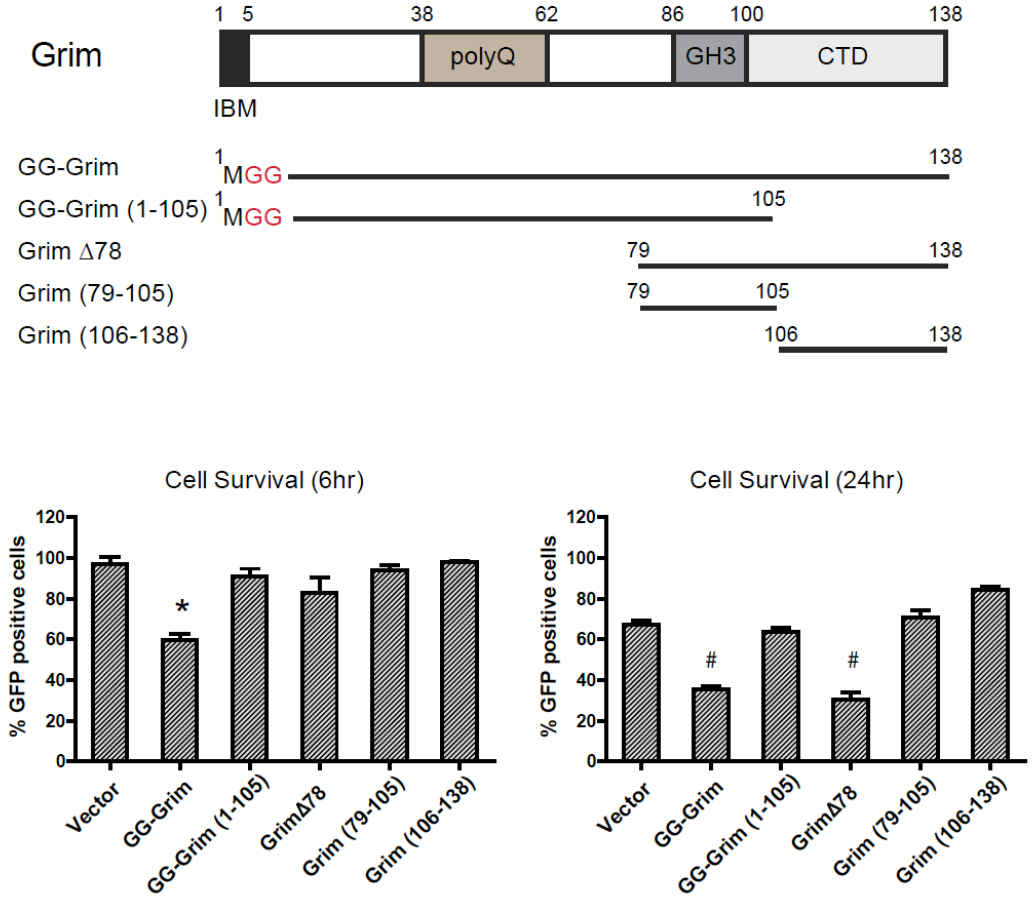

Fig. 4.2 Lethality of GG-Grim deletion mutants. S2 cells were transfected with 800 ng pRmHa3 plasmid (vector control, GG-Grim or indicated Grim deletion mutants) along with 100 ng pAc-GFP. After 16 h, cells were split in half and one portion was induced with $CuSO_4$, while the other was not. GFP positive cells were counted after 6 h or 24 h induction by flow cytometry. The ratio of GFP positive cells was used to calculate the cell survival rate. The data were collected from three individual experiments. [$*P < 0.05$, GG-Grim is significantly different from all the other group; $^{\#}P < 0.05$, GG-Grim and Grim Δ78 are significantly different from vector, GG-Grim (1-105), Grim (79-105), and Grim (106-138).]

4.3.3 Grim localization in S2 cells by fluorescence imaging

Claveria et al (2002) showed that Grim's localization is independent of its IBM and more related to the GH3 domain; however, the localization of the GH3 domain has remained controversial despite several follow-up studies (Chen et al., 2004; Freel et al., 2008; Olson et al., 2003a; Sandu et al., 2010; Thomenius et al., 2011). Claveria and co-workers (2002) reported that Grim localize to mitochondria due to its GH3 domain, while Chen and colleagues (2004) reported that the GH3 domain localizes to yet unidentified subcellular compartments. We decided to examine the localization of Grim, especially its C-terminus, by using immunofluorescence. We studied Grim's localization of Grim against the fluorescent mitochondrial marker MitoTracker®. We observed that Grim formed distinct punctae distributed throughout the cytosol, similar to what was previously reported (Claveria et al., 2002). Our data also revealed that the majority of Grim punctae did not colocalize with MitoTracker® (Fig. 4.3*A*). All of our experiments were performed in the presence of zVAD-fmk, in order to prevent cell death and cell detachment. MitoTracker® staining usually retains in cells after fixation even though this dye is somewhat dependent on mitochondrial potential (Chazotte, 2011). We also used a well-defined mitochondrial protein, dOmi, localized at mitochondrial intermembrane space, as a control to demonstrate our mitochondrial staining (Fig. 4.3*A*) (Challa et al., 2007). Indeed, the majority of Grim did not colocalize with mitochondria and the distribution was markedly different from dOmi-myc (Fig. 4.3*A*).

To visualize the localization of Grim in live cells, we expressed Grim as a GFP fusion protein. Notably, when GFP was placed at the N-terminus of Grim, it did not alter Grim's localization (Fig. 4.3*B*) or colocalize with MitoTracker®. We also observed that

the GH3 domain of Grim, while it had the ability to form punctae, also failed to colocalize with mitochondria (Fig. 4.3*B*). In addition to MitoTracker®, we also used other mitochondrial markers, such as mito-GFP, which contains the mitochondrial targeting sequence from cytochrome *c* oxidase, and dMFN, a mitochondrial fusion protein controlling mitochondrial dynamics. Similar to MitoTracker® staining, Grim failed to colocalize with either mitochondrial marker (Fig. 4.3*C*).

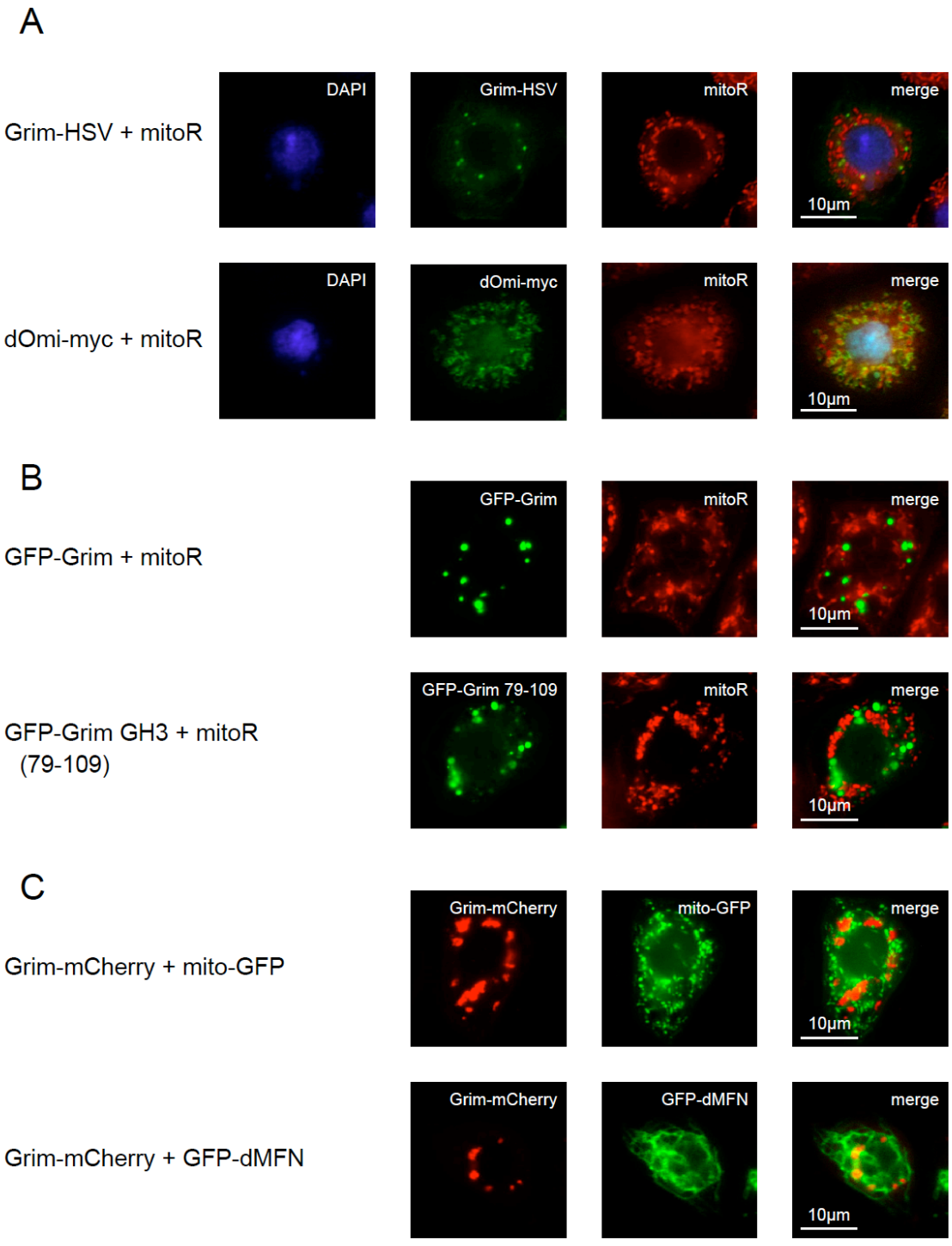

Fig. 4.3 The localization of Grim and dOmi in S2 cells. (*A*) S2 cells were transfected with pRmHa3-Grim-HSV or pRmHa3-dOmi-myc in the presence of zVAD-fmk (50 μM). Cells were then incubated with MitoTracker® Red prior to paraformaldehyde fixation, and stained with rabbit anti-HSV or anti-myc antibody. DNA was stained with Hoechst 33342, and a rabbit secondary antibody conjugated with FITC was used to detect Grim or dOmi expression. (*B*) S2 cells were transfected with pIE-GFP-Grim or pIE-GFP-Grim GH3 (amino acid 79-109), and cells were incubated with MitoTracker® Red prior to live cell imaging. (*C*) S2 cells were cotransfected with pIE-Grim-mCherry and an mitochondrial marker, mito-GFP or GFP-dMFN. Scale bar: 10 μm.

4.3.4 Grim forms cytosolic punctae

Upon discovery of the cytosolic Grim punctae, we searched the literature and discovered some interesting reports showing that mitochondria are associated with mRNA processing (P)-bodies and have the ability to modulate miRNA function (Bandiera et al., 2011; Huang et al., 2011). The fact that Grim is capable of inducing translational repression *in vitro* (Yoo et al., 2002) and that it does not colocalize with mitochondria, but remains proximal to mitochondria led us to speculate that Grim might in fact associate with P-body granules. We examined whether Grim colocalized with a variety of mRNA-protein (mRNP) complexes, including P-bodies and stress granules (Fig. 4.4). Decapping proteins, such as DCP1, localize to P-bodies, often near mitochondria and are commonly used as P-body markers (Huang et al., 2011). As speculated, we found that florescence-tagged Grim colocalized with P-body components, DCP1 and Me31B, but not with a common mRNP granule component, dFMR1, or stress granule components, Rox-8 (TIA-1 homolog in *Drosophila*) and eIF4E, which binds the cap of mRNA and accumulates in granules when cells are stressed (Fig. 4.4). Our data also revealed that the both N- and C- terminal tagged Grim colocalize with DCP1, indicating that Grim localizes to P-bodies in an IBM-independent manner (Fig. 4.4). We decided to use N-terminal tagged Grim for the following colocalization study in order to investigate IBM-independent cell death without the possible interaction from DIAP1.

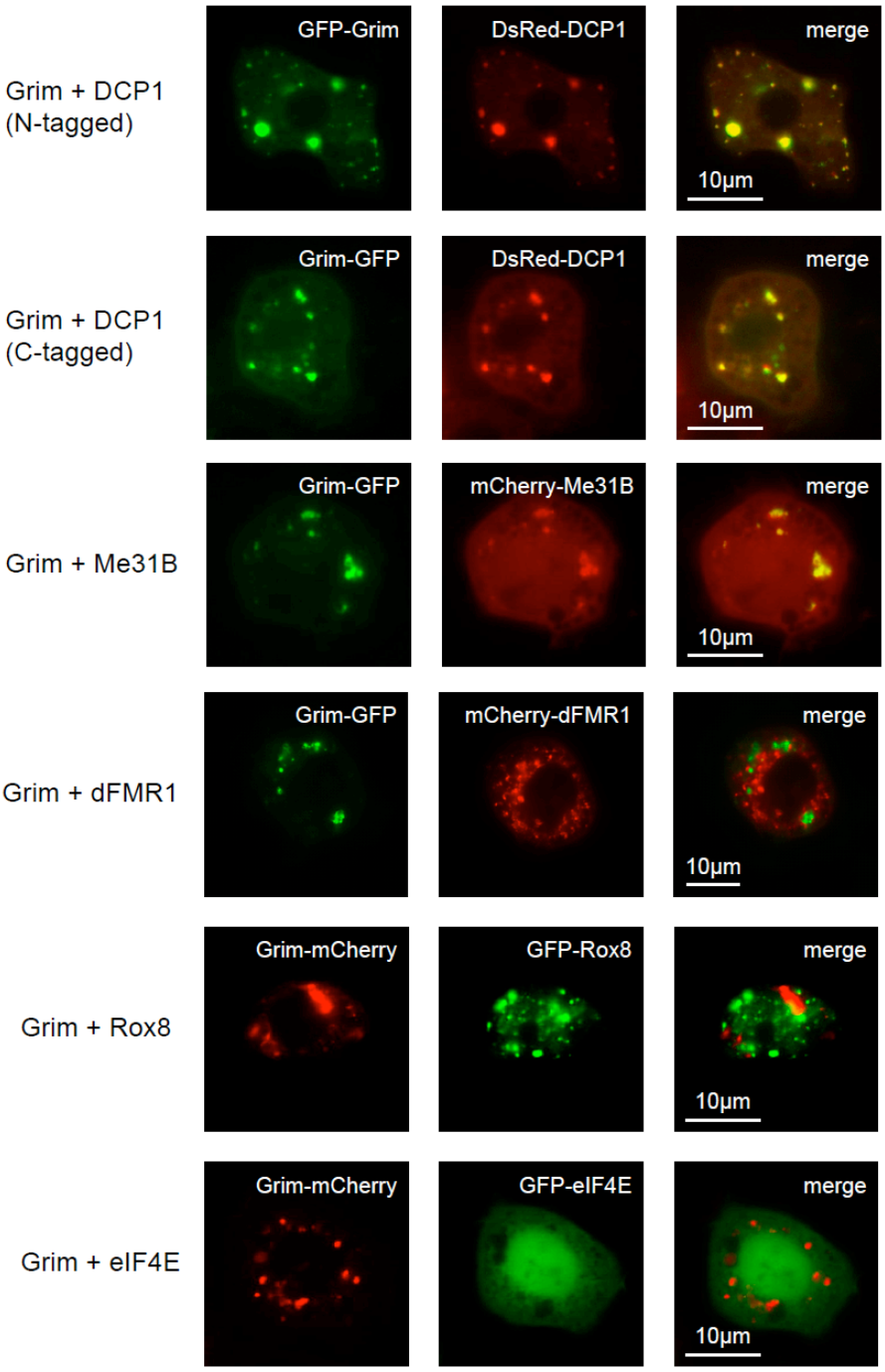

Fig. 4.4 Grim colocalizes with DCP1 and Me31B, but not dFMR1 or stress granule markers. S2 cells were cotransfected with fluorescent-tagged Grim along with a fluorescent-tagged P-body component or stress granule component in the presence of zVAD-fmk (50 μM). Scale bar: 10 μm.

The colocalization of Grim and DCP1 was dramatic; therefore, we next utilized deletion analysis to identify the minimum region(s) of Grim necessary for the colocalization. Grim possess a polyQ region, and glutamines are account for 21% of the protein (29 glutamines in total 138 amino acids). Depending upon the length of the polyQ tract, PolyQ repeat expansion prone to aggregate and cause toxicity to cells (Scherzinger et al., 1997; Shao and Diamond, 2007). N-terminal fragments of Grim comprising 1-85 , however, exhibited a cytosolic distribution, despite containing the polyglutamine expansion (aa 38-62). Surprisingly, we found that a C-terminal region (110-124), just downstream of the GH3, was responsible for the colocalization of Grim with DCP1 (Fig. 4.5; compare 1-109 with 1-124). Interestingly, the sequence from amino acid 110-118 (TVIQISFEI) is 100% identical among all twelve sequenced *Drosophila* species. We therefore expressed this C-terminal fragment (GFP-Grim 110-124) to further study its function. While this fragment did not form distinct punctae when expressed alone, it was readily recruited to DCP1 granules when coexpressed with DCP1 (Fig. 4.6). Importantly, GFP-Grim (79-109), which encompasses the GH3 domain, did not colocalize with DCP1, but this region still forms punctae independent of DCP1 or P-bodies. Moreover, GFP-Grim (79-109) exhibited more cytosolic distribution than GFP-Grim (1-109), indicating that the first 78 amino acids enhanced formation of the Grim punctae (Fig. 4.5 and Fig. 4.6). GFP-Grim (125-138) was not affected by DCP1 coexpression, and effectively served as a negative control. Collectively, the data indicated that the GH3 domain in Grim was responsible for punctae formation and a C-terminal (and previously uncharacterized) domain was responsible for recruiting Grim to DCP-1 granules. Immunoprecipitation experiments were also performed to verify the

interaction between Grim and DCP1, and the results confirmed that Grim (110-124) fragment was sufficient to interact with DCP1 (Fig. 4.7). However, the binding of Grim (110-124) was clearly weaker than that of full-length Grim (Fig. 4.7, lane 2 and 3). This result is consistent with the fact that Grim dimerizes in cells (Fig. 3.7*B*) and that a tripartite interaction between dimerized Grim and DCP1-containing P-bodies is likely to be much stronger than a single Grim-DCP1 interaction alone. Finally, to our surprise, the C-terminus of Grim is important for both its localization to P-bodies (Fig. 4.5 and 4.6) and its killing activity (Fig. 4.2); therefore, we concluded that localization to P-bodies is required for IBM-independent cell killing, perhaps due to increased translational repression.

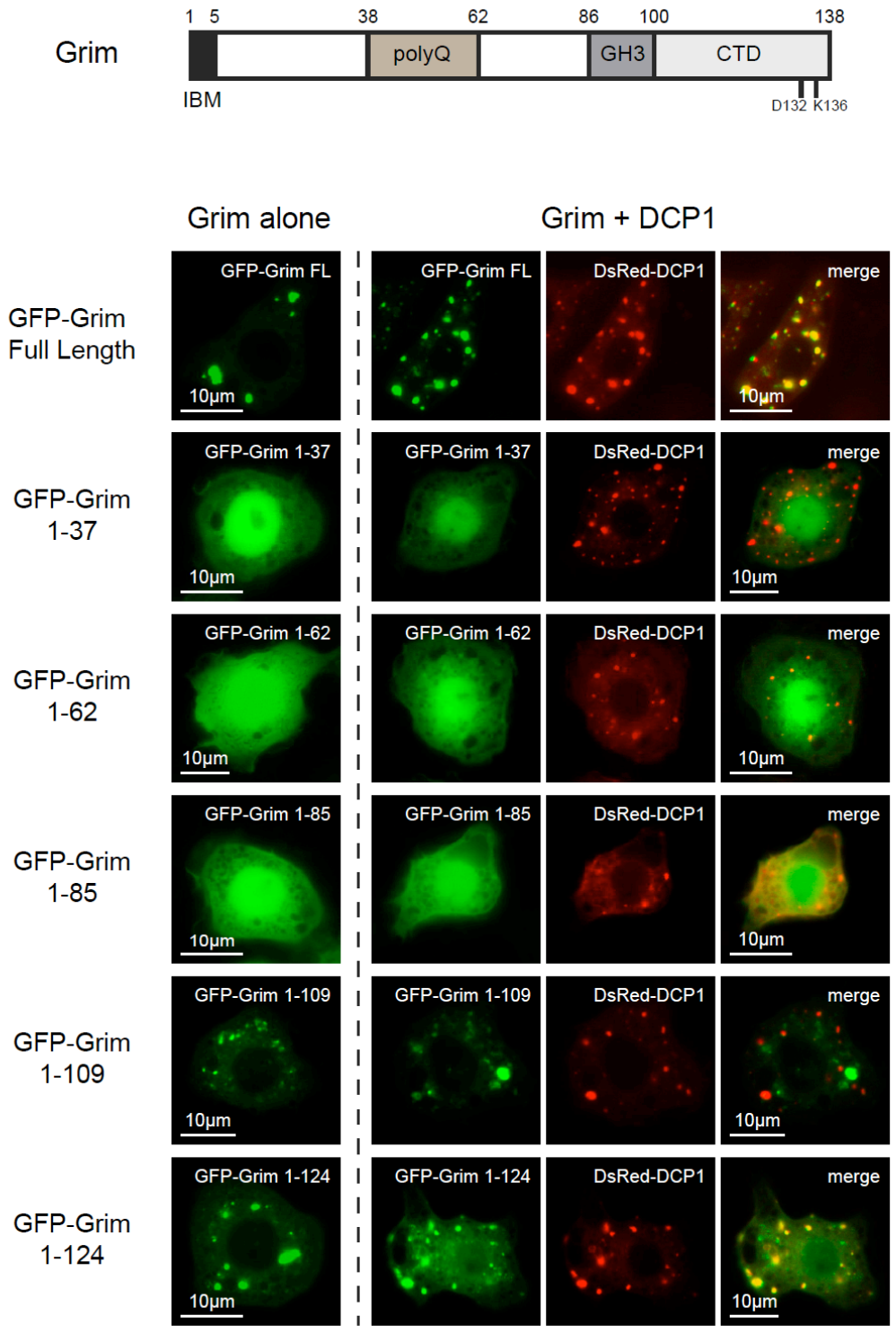

Fig. 4.5 Grim and Grim (1-124) colocalize with DCP1. S2 cells were cotransfected with pIE-DsRed-DCP1 and GFP-tagged Grim or one of its deletion mutants in the presence of zVAD-fmk (50 μM). Scale bar: 10 μm.

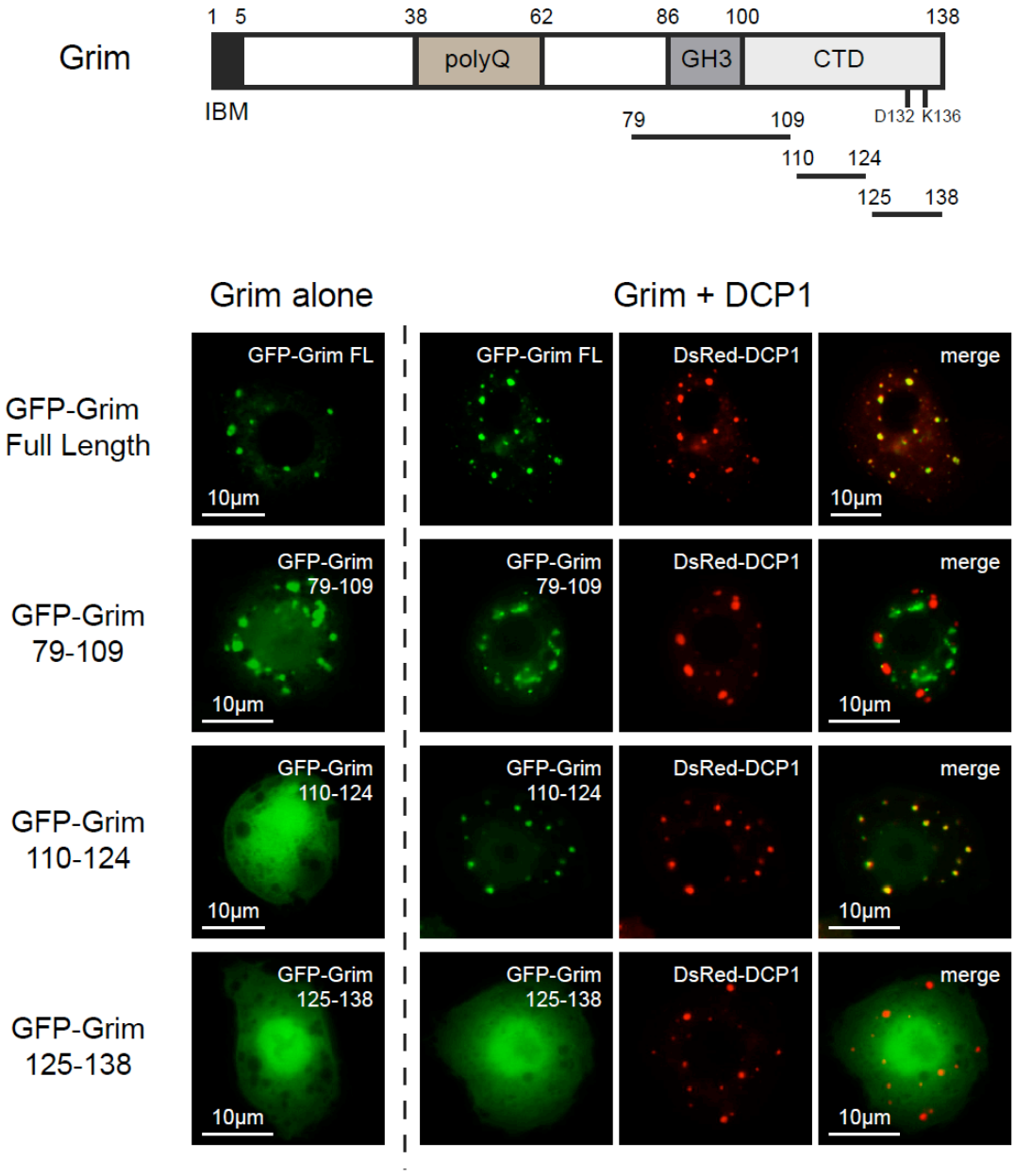

Fig. 4.6 Grim forms punctae via its GH3 domain and colocalizes with DCP1 via a C-terminal (110-124) fragment. S2 cells were cotransfected with pIE-DsRed-DCP1 and GFP-tagged Grim or one of its deletion mutants in the presence of zVAD-fmk (50 μM). Scale bar: 10 μm.

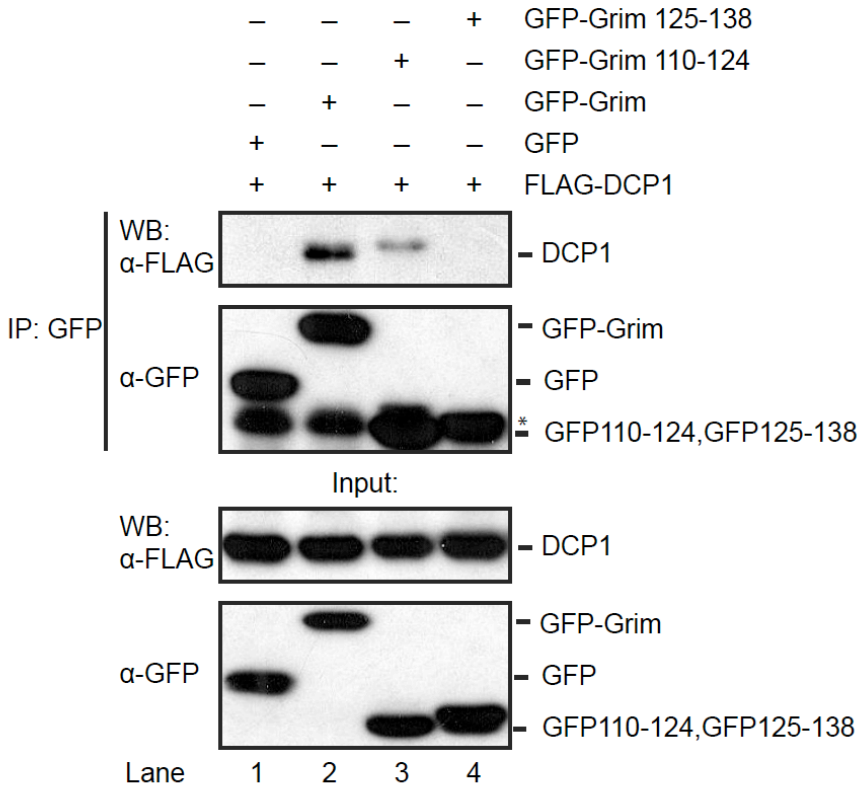

Fig. 4.7 Grim and Grim (110-124) interact with DCP1. S2 cells were cotransfected with pIE-Flag-DCP1 and GFP-tagged Grim or one of its deletion mutants in the presence of zVAD-fmk (50 μM). Immunopreicipitation was performed with an anti-GFP antibody. Grim complexes were analyzed by Western blotting. Note: the antibody light chain largely overlapped with GFP (110-124) and GFP (125-138).

4.3.5 The P-body component Me31B is important for Grim's activity

We performed RNAi on several P-body components to investigate whether any of them participated in Grim-mediated cell death. The data revealed that Me31B knockdown reduced Grim-induced cell death, but almost completely prevent GG-Grim-induced cell death (Fig. 4.8*A*). Knockdown of other P-body components, GW-182, or combined DCP1 and DCP2, had no impacts on Grim's activity (Fig. 4.8*B*). Overall, our data indicates that IBM-independent cell death occurs in an Me31B-dependent manner, most likely through translational repression because Me31B was often reported to inhibit translation in flies (Barbee et al., 2006; Hillebrand et al., 2010; Nakamura et al., 2001).

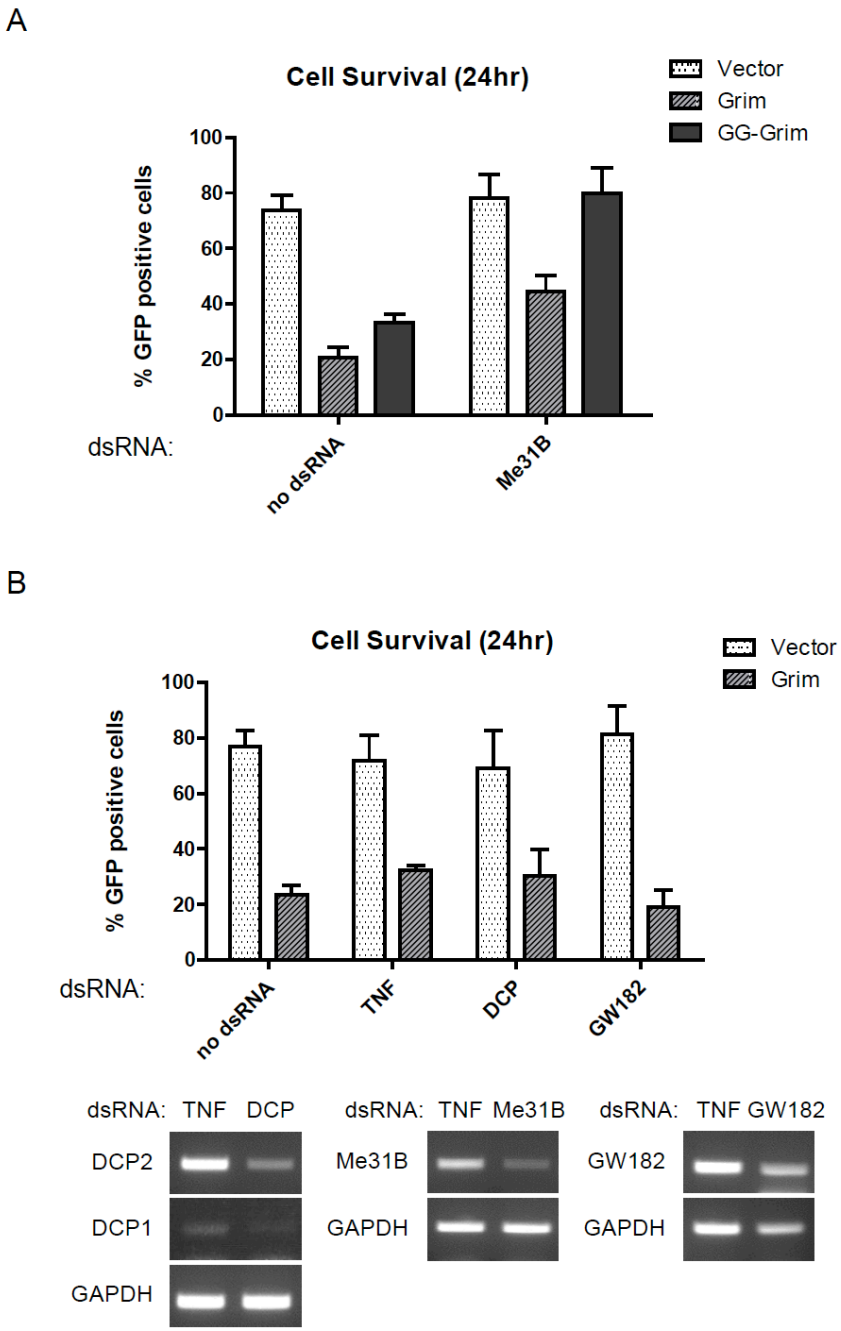

Fig. 4.8 The effect of P-body components on Grim-induced cell death. (*A*) S2 cells were pretreated with no dsRNA or dsMe31B for 48 h. S2 cells were then transfected with 800 ng pRmHa3-HSV, pRmHa3-Grim-HSV, or GG-Grim along with 100ng pAc-GFP. After induction, cells were collected at 24 h by flow cytometry. (*B*) S2 cells were treated with no dsRNA, dsTNF (control), dsDCP1 and DCP2, or dsGW182 for 48h. S2 cells were then transfected with 800ng pRmHa3-HSV or pRmHa3-Grim-HSV along with 100ng pAc-GFP. GFP positive cells were counted after 24 h induction by flow cytometry. The ratio of GFP positive cells was used to calculate cell survival rate. RT-PCR was performed to verify the knockdown efficiency of Me31B, DCPs, or GW182. The data were collected from three individual experiments.

4.3.6 Me31B interacts with Grim independent of RNA

Due to the fact that Me31B is involved in IBM-independent cell death and that Me31B in fact colocalizes with Grim (Fig. 4.4), we aim to verify whether Me31B and Grim form a functional complex. P-body components form complexes with one another usually in a direct, indirect or RNA-dependent manner (Parker and Sheth, 2007). We examined the interaction between Grim and Me31B and found that it did not require RNA, since the addition of RNase did not disrupt binding (Fig. 4.9, lane 2 and 4). Overall, we have demonstrated that IBM-independent cell death requires Grim's C-terminus to localize to DCP1-containing granules (P-bodies) (Fig. 4.5, 4.6. and 4.7) as well as a P-body localized translational regulator, Me31B (Fig. 4.8 and 4.9).

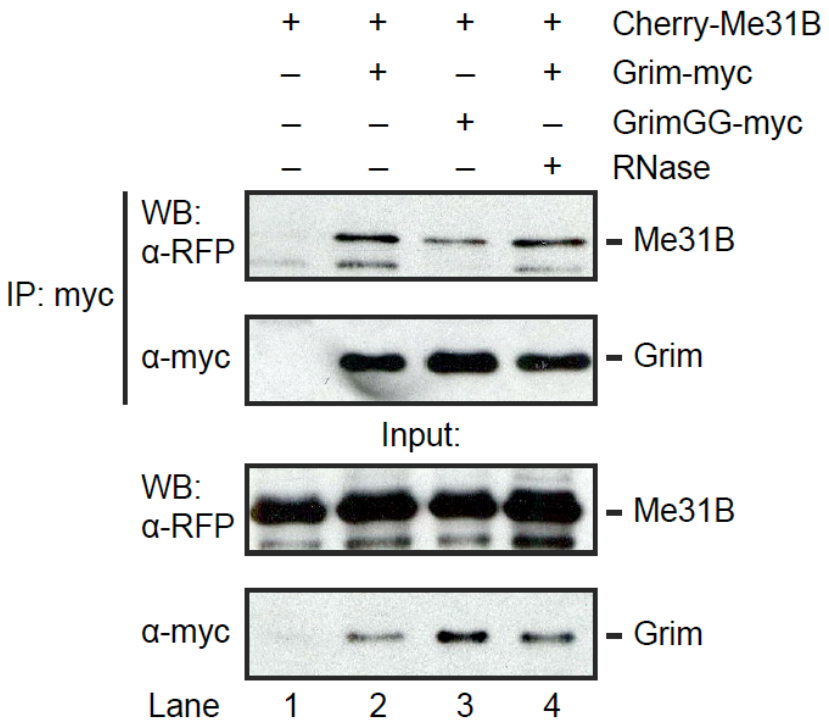

Fig. 4.9 The interaction between Grim and Me31B is RNA-independent. S2 cells were transfected with 500 ng pRmHa3 plasmid DNA, pRmHa3-Grim-myc, or GG-Grim-myc along with 500 ng pIE-mCherry-Me31B. After induction, lysates were prepared in buffers with or without RNase. Immunoprecipitation was performed using an anti-myc antibody, and Grim complexes were analyzed by immunoblotting.

4.4 Discussion

In this chapter, we have demonstrated that Grim has a bi-functional killing effect through the IBM region (N-terminus) as well as the C-terminus. As previously reported, we observed that Grim formed distinct punctae distributed throughout cytosol, but the actual identity of the punctae was controversial (Chen et al., 2004; Claveria et al., 2002). We cannot completely rule out the possibility that some Grim localizes to mitochondria and induces mitochondria insult, but it is clear that the majority of Grim does not localize to mitochondria. The fact both Grim and Reaper have IBM-independent killing effects and both proteins contain the GH3 domain had drawn a lot of attention from various groups (Chen et al., 2004; Claveria et al., 2002; Freel et al., 2008; Olson et al., 2003a; Sandu et al., 2010; Thomenius et al., 2011). Indeed, the GH3 domain has the ability to form large distinct punctae, and deletion or mutation of GH3 impairs IBM-independent killing, but the functions of the remainder of both proteins in uncharacterized (Claveria et al., 2002; Olson et al., 2003a). Chen and co-workers (2004) partially characterized the IBM-independent activity of Reaper, but were unable to identify the nature of the punctae. Interestingly, we identified that the C-terminus of Grim, especially amino acids 110-124 was important for directing Grim to P bodies. Even though this fragment does not in Reaper, it is conserved within Grim in all sequenced *Drosophila* species. We are still unclear what causes the punctae formation in the GH3 domain, but without the C-terminus, Grim fails to localize to P-bodies or to induce cell death. We have provided evidence to demonstrate that C-terminus and the GH3 domain are equally important for IBM-independent cell death, but neither domain alone is sufficient to kill, implying that Grim needs to localize to the right spot to induce IBM-independent cell death.

We used an overexpression system to visualize P-bodies or stress granules, but were aware that the size or distribution of these granules might be affected. Even so, the colocalization of Grim was selective for P-body components, such as DCP1 and Me31B, but not dFMR1, or the stress granule components, eIF4E and ROX8. Furthermore, in loss of function experiments, we clearly demonstrated that loss of Me31B reduced Grim-induced cell death, confirming genetic interaction between *grim* and *me31b* at endogenous levels.

Yoo et al. (2002) showed that recombinant Grim is capable of inducing general translational inhibition *in vitro*, but the mechanism is not defined. We found knockdown of Me31B reduced Grim-induced cell death, suggesting that Grim might involve in Me31B function as a translational suppressor. It is unclear whether Grim-induced translational inhibition is a general or transcript-specific effect, or whether Grim participates in miRNA-dependent inhibition. It is possible that Grim suppresses the translation of an anti-apoptotic mRNA transcript, such as *diap1* or activates the translation of a proapoptotic protein. Alternatively, Grim might act as a general translational repressor, similar to Reaper, by interacting with ribosomal small subunits to disrupt cap-dependent translation (Colon-Ramos et al., 2006). In the latter scenario, once cap-dependent translation is compromised, short live proteins, such as DIAP1, will reduce in cells (Yoo et al., 2002). Cell death occurs once DIAP1 protein drops below a certain threshold, so that it is no longer able to inhibit caspase activation.

DCP2 is the main enzyme for catalyzing decapping, but it requires DCP1 and other proteins to stabilize the decapping enzyme complex (Bail and Kiledjian, 2006; Chang et al., 2014; She et al., 2008). Even though knockdown of DCP1 was marginal

(Fig. 4.8), reduction in the main decapping enzyme (DCP2) should still impair decapping activity. However, reduction in decapping enzymes had limited effect on Grim-induced cell death, suggesting that Grim does not kill by enhancing decapping activity, but we cannot rule out that Grim might inhibit decapping activities (if it stabilizes the mRNA of a proapoptotic gene). The effect of DCP1 knockdown needs to be further characterized due to the insufficient efficiency in this study. The fact that P-body component Me31B forms a complex with Grim to participate in IBM-independent killing (Fig 4.4, 4.8 and 4.9) and that the killing requires Grim's C-terminus, which also directs Grim to localize to DCP1-containing granules (Fig. 4.5, 4.6. and 4.7), strongly suggested that Grim might form a complex with DCP1 and Me31B to perform its function. In fact, Dhh1 (yeast Me31B) has been reported to interact with decapping as well as deadenylase complexes (Coller et al., 2001). Due to the complexity in P-bodies, we cannot rule out that other protein components, such as mRNA binding proteins, the LSM exonuclease complexes (which interacts with DCP1), or deadenylase complexes might also participate in IBM-independent cell death (Braun et al., 2012; Chang et al., 2014; Coller et al., 2001; Jinek et al., 2008; Tritschler et al., 2009; Tritschler et al., 2008).

dFMR1 is a common RNA granule component and can shuttle between the cytosol and nucleus, as well as the polysome and P-bodies (Darnell et al., 2011). It is an RNA-binding protein particularly important in repressing differentiation in neurons (Bassell and Warren, 2008; Darnell et al., 2011; Kim et al., 2009). The fact that Grim colocalizes with Me31B and DCP1, but not with dFMR1, indicates that Grim/DCP1/Me31B form unique granules to selectively associate with certain P-body components. EDC3 and Tral have mutually exclusive interactions with Me31B

(Tritschler et al., 2008), but it is unclear whether this is also the case for Grim and dFMR1. Further experiments are needed to decipher the components of Grim/DCP1/Me31B granules, and how they work together to induce cell death through translational regulation.

CHAPTER 5

Concluding remarks and future directions

Programmed cell death has been studied extensively in many organisms and is highly conserved from *C. elegans* to human. In flies and mammals, cell death can be regulated by altering the balance among caspases, IAPs, and IAP antagonists. IAPs prevent caspase-mediated cell death not only through direct binding and inhibition, but also by directly ubiquitinating caspases, which inactivates them or targets them for degradation (Ditzel et al., 2008; Lee et al., 2011; Li et al., 2011; Muro et al., 2002; Wilson et al., 2002; Yan et al., 2004). IAP antagonists promote cell death by displacing IAP-bound caspases as well as by promoting IAP autoubiquitination (Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). While the role of caspases in mediating cell death in multicellular organisms has been studied for the past two decades, it is still unclear how cell death activators/enhancers, such as IAP antagonists, are regulated at the molecular level.

We used Grim to study the regulation of IAP antagonists-induced cell death, and we identified that Grim induces cell death through two distinct mechanisms. We discovered that IBM-dependent cell death induced by Grim was regulated by a novel regulation between both caspases and the proteasome system, while IBM-independent cell death was achieved by Grim's distinct cellular localization, which leads to translational repression. In both cases, caspases are activated, but DIAP1 physically interacts with Grim only in the former case, the IBM-dependent manner. In our studies,

Grim activates caspase activity by utilizing its IBM to displacing active DRONC and DrICE from DIAP1, but is itself susceptible to both caspase cleavage and DIAP1-mediated ubiquitination, with the former subverting the latter (Fig. 5.1) (Yeh and Bratton, 2014). DIAP1 utilized UbcD1 to ubiquitinate Grim at Lys136 at its C-terminus. We discovered that Grim dimerizes, engages the BIR1 and BIR2 domains in DIAP1, and thus forms a tripartite complex. Remarkably, however, DIAP1-UbcD1 ubiquitinates only the BIR2-bound Grim, demonstrating unique structural selectivity and raising the possibility that BIR1-bound Grim may be degraded through an alternative mechanism. Regardless, Grim is also cleaved by DrICE at Asp132, which removes the lysine necessary for DIAP1-dependent ubiquitination of Grim (Fig. 5.1) (Yeh and Bratton, 2014). DrICE cleaves Grim, either prior to or following ubiquitination, resulting in a form of Grim that is no longer ubiquitinated or degraded. The accumulation of Grim in cells then displaces even more active caspases from DIAP1, initiating a caspase amplification loop that results in greater caspase activity and increased cell death.

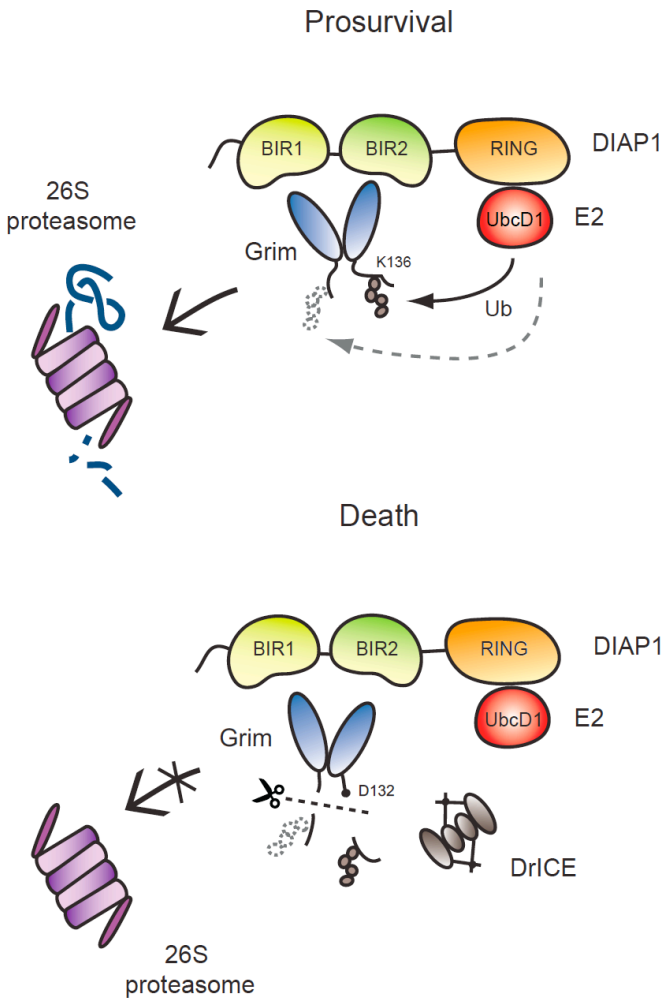

Figure 5.1 Regulation of Grim by DIAP1 and caspases. Grim interacts with DIAP1 through its BIR1 and BIR2 domains. BIR2-bound Grim is ubiquitinated on Lys136, so that when Grim levels are relatively low, most Grim is ubiquitinated and degraded by the proteasome (prosurvival). However, at higher Grim levels, DrICE is activated and cleaves Grim at Asp132, liberating its C-terminus (and any ubiquitin chains) and facilitating a caspase amplification loop. (Adapted from Yeh and Bratton, 2014)

Our findings demonstrate that an important crosstalk exists at the substrate level between caspases and the proteasomal system. In fact, any protein with a single lysine (or a critical lysine responsible for its stability) could undergo a similar type of regulation as described here, if flanked by a caspase cleavage site. Moreover, this dual regulation of a protein substrate by a protease and E3 ligase need not be limited to caspases or even cell death regulation. In our hands, we have identified that one mammalian IAP antagonist, ARTS, might be regulated through a similar way as Grim. ARTS inhibits XIAP function and is required for hair follicle stem cell apoptosis (Fuchs et al., 2013). It interacts with XIAP through an unconventional IBM located at its C-terminus (Larisch et al., 2000). XIAP is able to ubiquitinate ARTS at its N-terminal lysine (K3) (Bornstein et al., 2012). We have noticed that there is a potential caspase cleavage site (FLED) downstream of the lysine (Fig. 5.2). When recombinant caspase-3 was added into the HEK293T cells lysates with ARTS or ARTS mutants ectopically overexpressed, we observed a band shift on wild-type ARTS, and the lysine mutant K3R, but not the cleavage site mutant D8A (Fig. 5.2). The result was promising, and we are still investigating the function and regulation of ARTS cleavage.

We have successfully demonstrated a unique interplay between caspases and the proteasome system using Grim and the IBM-dependent recruitment of DIAP1 as an example. However, in addition to IBM-dependent cell death, some IAP antagonists possess a second mechanism to induce cell death, but this mechanism is often neglected or unclear (Challa et al., 2007; Chen et al., 2004; Claveria et al., 2002; Olson et al., 2003a). How Grim induces cell death without its IBM has always been controversial, and several possibilities were proposed (Chen et al., 2004; Claveria et al., 2002; Claveria

et al., 2004). The GH3 domain seems to partially contribute to IBM-independent cell death in a mitochondria-dependent manner (Claveria et al., 2002; Claveria et al., 2004), even though there is discrepancy about whether Grim localizes to mitochondria (Chen et al., 2004). Grim was also reported to induce translational repression *in vitro* (Yoo et al., 2002), but mechanistically how Grim might induce translational repression *in vivo* was never investigated. In this study, we have demonstrated that both GH3 and the C-terminus are required for this activity. Given that the Grim (110-124) region directs Grim to P-bodies and that knockdown of Me31B reduced Grim-induced cell death, it suggests that IBM-independent cell death occurs, at least in part, through Me31B-mediated translational repression.

There are still unanswered questions to unveil the IBM-independent mechanism of Grim on translational repression. We are still uncertain whether IBM-independent cell death is carried out by inhibiting (or enhancing) specific mRNA transcripts or by general translational repression. If Grim-induced death is transcript-specific, identifying the mRNA transcript(s) and the mechanism by which it is recruited to the Grim/DCP1/Me31B complex will be an important task in the future. If Grim-induced translational repression is a general effect, Grim would target common factors regulating protein translation, such as initiation factors, elongation factors, or proteins regulating termination. In addition, whether other P-body components participate in IBM-independent cell death remains unclear. RNA granules usually suppress mRNA translation or regulate mRNA stability through intricate protein complexes. Decapping machinery is usually coupled with exonuclease complex as well as deadenylase complexes in P-bodies (Aizer and Shav-Tal, 2008; Braun et al., 2012; Chang et al., 2014;

Eulalio et al., 2007a; Tritschler et al., 2008). Since none of the Grim/DCP1/Me31B granule components contain RNA-binding motifs, it is very likely that at least an mRNA-binding protein is involved in the translational repression process.

Notably, we hypothesized that the translational repression activity of Grim is IAP-independent, since the IBM region of Grim is neither required for its localization within P-bodies nor its interaction with DCP1 and Me31B within the P-body. However, we cannot rule out that IAPs might participate in P-body-related functions. It is possible that after Grim is recruited to P-bodies, IAPs might also be recruited and brought into the complex in order to ubiquitinate Grim or other components in the P-bodies. Strikingly, we have discovered that when Grim is coexpressed with DIAP1, it increases the size of Grim punctae and induces formation of enlarged ring-like structures, but they still do not colocalize with mitochondria (Fig. 5.3). Further study will be required to understand whether the ring-like structures are due to ubiquitination, and if so, whether specific ubiquitin chains are generated. These studies would open a brand new field if IAPs are recruited to P-bodies and modify P-body function.

Our results are consistent with a model that Grim induces caspase activation through two distinct mechanisms: one requires the N-terminus of Grim (IBM-dependent) and the other requires its C-terminus (IBM-independent). An obvious question is whether should such dual mechanisms exist on the same protein molecule? Such an arrangement could ensure that maximum caspase activity is achieved at a particular point in time during development. Alternatively, these two mechanisms might be regulated coordinately to establish finer control over the degrees of caspase activity required for different biological needs. IBM-dependent cell death may occur when massive cell death

is required in short period of time, while at different stages of development, C-terminal-dependent caspase activation may be preferred to maintain low levels of caspase activity for a given biological function. In fact, Dronc is required for mediating both cell death (in dying cells) and apoptosis-induced proliferation (in undead cells), in which compensatory cell outgrowth occurs after inhibition of downstream effector caspases (Kondo et al., 2006).

The second question that comes to mind is whether there exists a crosstalk between the two caspase-activating mechanisms? Under which circumstances would Grim favor one mechanism over the other? In our study, we isolated IBM-independent cell death using GG-Grim or an N-terminally-tagged Grim in order to avoid interference from DIAP1, even though an isoform of Grim, devoid of its IBM domain, has not been detected *in vivo*. However, the IBM function may be covered through the following model. In our lab, we have identified that the chaperone protein HSC70 interacts with Grim (data not shown), and it has been reported that the E3 ligase, C-terminal Hsc70-interacting protein (CHIP), forms a complex with HSC70 to ubiquitinate its client proteins (Ahmed et al., 2012; Gao et al., 2010; Smith et al., 2013). According to a previous report, proteins with polyglutamine expansion have a tendency to recruit the HSC70/CHIP complex (Jana et al., 2005). If Grim recruits the HSC70/CHIP complex through the polyglutamine domain in Grim, it is possible that Grim can be ubiquitinated by the HSC70/CHIP complex. Importantly, the polyglutamine region resides in the internal portion of Grim, so Grim would be subject to two potential ubiquitination sites, the N-terminus and the lysine at its C-terminus. An IBM mutant of Grim can therefore be generated if ubiquitination occurs at its N-terminus (Ub-Grim). If the speculated

model is true, the existence of Ub-Grim may act as a switch to selectively foster the IBM-independent pathway.

The sophisticated regulation of Grim through multiple mechanisms suggests that Grim has different functions other than promoting cell death. As a precedent, the mammalian IAP antagonist Omi has a biological role in regulating autophagy through its IBM-independent serine protease activity (Li et al., 2010). Indeed, Grim is present, and caspases are activated during the development of Malpighian tubules in the fly embryo, but they do not cause significant cell death (Tapadia and Gautam, 2011). During metamorphosis, sensory organ progenitors undergo asymmetric division and give rise to different organs, such as thoracic microchaete bristle and non-innervated bristle at the posterior wing margin. Grim is required to eliminate different descendants from the sensory precursor (Fig. 1.5), but it is unclear how Grim accurately kills the specific daughter cell (a glial cell, or a pIIb cell) without affecting the other daughter cell. One explanation is that Grim is only expressed in the "meant to be dead" cell, but not the other cell. Alternatively, Grim may be present in all of the daughter cells (to perform its physiological function), but is regulated differently after asymmetric cell division, so that only specific cells reach an apoptotic threshold. In the other live cell (or undead cells), Grim levels are kept low to perform specific functions or be eliminated through the regulatory system between caspases and the proteasome machinery.

It is worth noting that Grim is also ubiquitinated by another *Drosophila* IAP, DIAP2, through the interaction between Grim's IBM and the BIR domains in DIAP2. Unlike DIAP1, DIAP2 plays a major role in innate immunity and nuclear factor kappa B (NF-κB) signaling in flies. It often mediates K63-based ubiquitination of substrates, such

as IMD (Paquette et al., 2010), and K63-linked ubiquitin chains generally prevent protein turnover, mediate the recruitment of other proteins, and/or alter subcellular localization. Whether Grim undergoes K63-based ubiquitination by DIAP2 or functions as a signaling molecule is currently unknown. However, Grim exhibits a distinct subcellular localization, and removal of K63-linked chains by caspases could alter its function in cells, perhaps converting it from a signaling molecule to a killer.

The study of IBM-dependent cell death is important, because humans also possess IAP antagonists, such as Smac and Omi, sharing a conserved four amino acid sequence that enhances cell death. Based on the IBM region, Smac mimetics have been designed and are currently in use in clinical trials (Hennessy et al., 2013; Pluta et al., 2010). It is yet unclear whether the non-IBM region of Grim also induces a conserved cell death pathway, but most of the P-body components (and IAPs) are conserved from flies to mammals. With a better understanding of IBM-independent cell death, new insights maybe made into how cell death is executed. Apparent differences in flies may well point to previously unidentified but important nuances in existing pathways in humans. At this point, we appreciate the complexity of Grim-induced cell death, which might explain why Grim seems to be more potent than Reaper or Hid at inducing midline neuronal cell death in *Drosophila* embryos when ectopically expressed (Wing et al., 1998). With its regulation by caspases and the proteasome system, as well as its bifunctional killing activity, Grim is an intriguingly efficient, yet highly regulated, apoptosis inducer.

ARTS

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1   MIKRFLEDTTDDGELSKFVKDFSGNASCHPPEAKTWASRPQVPEPR
47  PQAPDLYDDDLEFRPPSRPQSSDNQQYFCAPAPLSPSARPRSPWGK
93  LDPYDSSEDDKEYVGFATLPNQVHRKSVKKGFDFTLMVAGESGLGK
139 STLVNSLFLTDLYRDRKLLGAEERIMQTVEITKHAVDIEEKGVRLR
185 LTIVDTPGFGDAVNNTECWKPVAEYIDQQFEQYFRDESGLNRKNIQ
231 DNRVHCCLYFISPFGHGYGPSLRLLAPPGAVKGTGQEHQGQGCH
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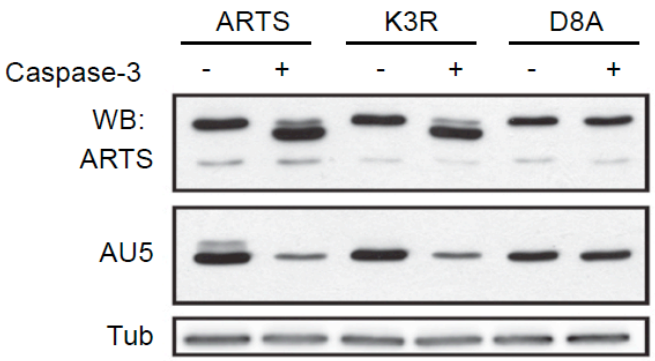

Figure 5.2 ARTS is cleaved by caspase-3 at Asp8. Mammalian IAP antagonist ARTS interacts with XIAP through its C-terminus, and is ubiquitinated by XIAP at lysine 3 (indicated in gray). ARTS possess FLED (underlined) sequence, which could serve as a caspase cleavage site. Recombinant caspase-3 was added into HEK293T cell lysates transfected with N-terminally AU5-tagged ARTS (pEF-AU5-ARTS), pEF-AU5-ARTS K3R (K3R), or pEF-AU5-ARTS D8A (D8A) for 30 mins at 37 °C. Lysates were then analyzed with Western blot.

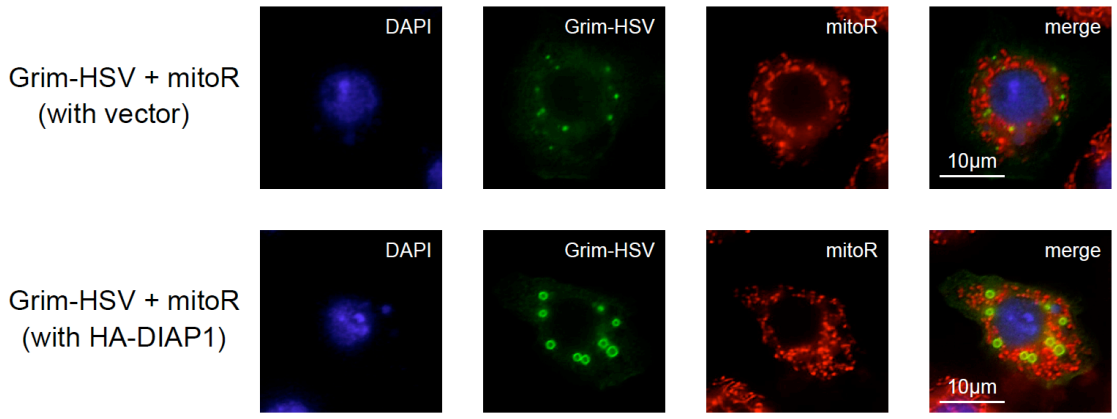

Figure 5.3 The localization of Grim in S2 cells. S2 cells were cotransfected with pRmHa3-Grim-HSV along with vector or pIE-HADIAP1 in the presence of zVAD-fmk. Cells were fixed with paraformaldehyde and stained with an anti-rabbit-HSV antibody. DNA was visualized with Hoechst 33342. Scale bar: 10 μm.

Reference

Abdelwahid, E., Yokokura, T., Krieser, R.J., Balasundaram, S., Fowle, W.H., and White, K. (2007). Mitochondrial disruption in Drosophila apoptosis. Dev Cell *12*, 793-806.

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X., and Akey, C.W. (2002). Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell *9*, 423-432.

Ahmed, S.F., Deb, S., Paul, I., Chatterjee, A., Mandal, T., Chatterjee, U., and Ghosh, M.K. (2012). The chaperone-assisted E3 ligase C terminus of Hsc70-interacting protein (CHIP) targets PTEN for proteasomal degradation. J Biol Chem *287*, 15996-16006.

Aizer, A., and Shav-Tal, Y. (2008). Intracellular trafficking and dynamics of P bodies. Prion *2*, 131-134.

Akao, Y., Marukawa, O., Morikawa, H., Nakao, K., Kamei, M., Hachiya, T., and Tsujimoto, Y. (1995). The rck/p54 candidate proto-oncogene product is a 54-kilodalton D-E-A-D box protein differentially expressed in human and mouse tissues. Cancer research *55*, 3444-3449.

Akao, Y., Matsumoto, K., Ohguchi, K., Nakagawa, Y., and Yoshida, H. (2006). Human DEAD-box/RNA unwindase rck/p54 contributes to maintenance of cell growth by affecting cell cycle in cultured cells. International journal of oncology *29*, 41-48.

Arama, E., Agapite, J., and Steller, H. (2003). Caspase activity and a specific cytochrome c are required for sperm differentiation in Drosophila. Dev Cell *4*, 687-697.

Arama, E., Bader, M., Srivastava, M., Bergmann, A., and Steller, H. (2006). The two Drosophila cytochrome C proteins can function in both respiration and caspase activation. EMBO J *25*, 232-243.

Bail, S., and Kiledjian, M. (2006). More than 1 + 2 in mRNA decapping. Nat Struct Mol Biol *13*, 7-9.

Bandiera, S., Hanein, S., Lyonnet, S., and Henrion-Caude, A. (2011). Mitochondria as novel players of the cellular RNA interference. The Journal of biological chemistry *286*, le19.

Barbee, S.A., Estes, P.S., Cziko, A.M., Hillebrand, J., Luedeman, R.A., Coller, J.M., Johnson, N., Howlett, I.C., Geng, C., Ueda, R.*, et al.* (2006). Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies. Neuron *52*, 997-1009.

Bassell, G.J., and Warren, S.T. (2008). Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron *60*, 201-214.

Besse, F., and Ephrussi, A. (2008). Translational control of localized mRNAs: restricting protein synthesis in space and time. Nature reviews Molecular cell biology *9*, 971-980.

Bornstein, B., Edison, N., Gottfried, Y., Lev, T., Shekhtman, A., Gonen, H., Rajalingam, K., and Larisch, S. (2012). X-linked Inhibitor of Apoptosis Protein promotes the degradation of its antagonist, the pro-apoptotic ARTS protein. The International Journal of Biochemistry & Cell Biology *44*, 489-495.

Braun, J.E., Truffault, V., Boland, A., Huntzinger, E., Chang, C.T., Haas, G., Weichenrieder, O., Coles, M., and Izaurralde, E. (2012). A direct interaction between DCP1 and XRN1 couples mRNA decapping to 5' exonucleolytic degradation. Nature structural & molecular biology *19*, 1324-1331.

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

Broemer, M., Tenev, T., Rigbolt, K.T.G., Hempel, S., Blagoev, B., Silke, J., Ditzel, M., and Meier, P. (2010). Systematic in vivo RNAi analysis identifies IAPs as NEDD8-E3 ligases. Mol Cell *40*, 810-822.

Buchan, J.R., and Parker, R. (2009). Eukaryotic stress granules: the ins and outs of translation. Mol Cell *36*, 932-941.

Cain, K., Brown, D.G., Langlais, C., and Cohen, G.M. (1999). Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. J Biol Chem *274*, 22686-22692.

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

Chai, J., Wu, Q., Shiozaki, E., Srinivasula, S.M., Alnemri, E.S., and Shi, Y. (2001). Crystal structure of a procaspase-7 zymogen: mechanisms of activation and substrate binding. Cell *107*, 399-407.

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

Challa, M., Malladi, S., Pellock, B.J., Dresnek, D., Varadarajan, S., Yin, Y.W., White, K., and Bratton, S.B. (2007). Drosophila Omi, a mitochondrial-localized IAP antagonist and proapoptotic serine protease. EMBO J *26*, 3144-3156.

Chang, C.T., Bercovich, N., Loh, B., Jonas, S., and Izaurralde, E. (2014). The activation of the decapping enzyme DCP2 by DCP1 occurs on the EDC4 scaffold and involves a conserved loop in DCP1. Nucleic acids research.

Chazotte, B. (2011). Labeling mitochondria with MitoTracker dyes. Cold Spring Harbor protocols *2011*, 990-992.

Chen, P., Ho, S.I., Shi, Z., and Abrams, J.M. (2004). Bifunctional killing activity encoded by conserved reaper proteins. Cell Death Differ *11*, 704-713.

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

Cheng, E.H.-Y., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K., and Hardwick, J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. Science *278*, 1966-1968.

Cheng, Z., Coller, J., Parker, R., and Song, H. (2005). Crystal structure and functional analysis of DEAD-box protein Dhh1p. RNA *11*, 1258-1270.

Choi, Y.E., Butterworth, M., Malladi, S., Duckett, C.S., Cohen, G.M., and Bratton, S.B. (2009). The E3 ubiquitin ligase cIAP1 binds and ubiquitinates caspase-3 and -7 via unique mechanisms at distinct steps in their processing. J Biol Chem *284*, 12772-12782.

Chu, C.Y., and Rana, T.M. (2006). Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoS biology *4*, e210.

Claveria, C., Caminero, E., Martinez, C., Campuzano, S., and Torres, M. (2002). GH3, a novel proapoptotic domain in Drosophila Grim, promotes a mitochondrial death pathway. Embo J *21*, 3327-3336.

Claveria, C., Martinez, A.C., and Torres, M. (2004). A Bax/Bak-independent mitochondrial death pathway triggered by Drosophila Grim GH3 domain in mammalian cells. The Journal of biological chemistry *279*, 1368-1375.

Coller, J.M., Tucker, M., Sheth, U., Valencia-Sanchez, M.A., and Parker, R. (2001). The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. RNA *7*, 1717-1727.

Colon-Ramos, D.A., Irusta, P.M., Gan, E.C., Olson, M.R., Song, J., Morimoto, R.I., Elliott, R.M., Lombard, M., Hollingsworth, R., Hardwick, J.M*., et al.* (2003). Inhibition of translation and induction of apoptosis by Bunyaviral nonstructural proteins bearing sequence similarity to reaper. Molecular biology of the cell *14*, 4162-4172.

Colon-Ramos, D.A., Shenvi, C.L., Weitzel, D.H., Gan, E.C., Matts, R., Cate, J., and Kornbluth, S. (2006). Direct ribosomal binding by a cellular inhibitor of translation. Nature structural & molecular biology *13*, 103-111.

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

Cutforth, T., and Gaul, U. (1999). A methionine aminopeptidase and putative regulator of translation initiation is required for cell growth and patterning in Drosophila. Mech Dev *82*, 23-28.

Darnell, J.C., Van Driesche, S.J., Zhang, C., Hung, K.Y., Mele, A., Fraser, C.E., Stone, E.F., Chen, C., Fak, J.J., Chi, S.W.*, et al.* (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell *146*, 247-261.

de Valoir, T., Tucker, M.A., Belikoff, E.J., Camp, L.A., Bolduc, C., and Beckingham, K. (1991). A second maternally expressed Drosophila gene encodes a putative RNA helicase of the "DEAD box" family. Proc Natl Acad Sci U S A *88*, 2113-2117.

Demontis, S., Rigo, C., Piccinin, S., Mizzau, M., Sonego, M., Fabris, M., Brancolini, C., and Maestro, R. (2006). Twist is substrate for caspase cleavage and proteasome-mediated degradation. Cell Death Differ *13*, 335-345.

Deveraux, Q.L., Leo, E., Stennicke, H.R., Welsh, K., Salvesen, G.S., and Reed, J.C. (1999). Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. Embo J *18*, 5242-5251.

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

Ditzel, M., Broemer, M., Tenev, T., Bolduc, C., Lee, T.V., Rigbolt, K.T., Elliott, R., Zvelebil, M., Blagoev, B., Bergmann, A.*, et al.* (2008). Inactivation of effector caspases through nondegradative polyubiquitylation. Mol Cell *32*, 540-553.

Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E., and Meier, P. (2003). Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. Nat Cell Biol *5*, 467-473.

Domingues, C., and Ryoo, H.D. (2012). Drosophila BRUCE inhibits apoptosis through non-lysine ubiquitination of the IAP-antagonist REAPER. Cell Death Differ *19*, 470-477.

Dorstyn, L., and Kumar, S. (2008). A biochemical analysis of the activation of the Drosophila caspase DRONC. Cell Death Differ *15*, 461-470.

Dorstyn, L., Mills, K., Lazebnik, Y., and Kumar, S. (2004). The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in Drosophila cells. The Journal of cell biology *167*, 405-410.

Dorstyn, L., Read, S., Cakouros, D., Huh, J.R., Hay, B.A., and Kumar, S. (2002). The role of cytochrome c in caspase activation in Drosophila melanogaster cells. The Journal of cell biology *156*, 1089-1098.

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

Dueber, E.C., Schoeffler, A.J., Lingel, A., Elliott, J.M., Fedorova, A.V., Giannetti, A.M., Zobel, K., Maurer, B., Varfolomeev, E., Wu, P.*, et al.* (2011). Antagonists induce a conformational change in cIAP1 that promotes autoubiquitination. Science *334*, 376-380.

Eckelman, B.P., and Salvesen, G.S. (2006). The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. J Biol Chem *281*, 3254-3260.

Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, M., Ghayur, T., Wong, W.W., Kamen, R., Weichselbaum, R.*, et al.* (1995). Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. Embo J *14*, 6148-6156.

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

Ernoult-Lange, M., Benard, M., Kress, M., and Weil, D. (2012). P-bodies and mitochondria: which place in RNA interference? Biochimie *94*, 1572-1577.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007a). P bodies: at the crossroads of post-transcriptional pathways. Nature reviews Molecular cell biology *8*, 9-22.

Eulalio, A., Behm-Ansmant, I., Schweizer, D., and Izaurralde, E. (2007b). P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol *27*, 3970-3981.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. Journal of immunology *148*, 2207-2216.

Feltham, R., Bettjeman, B., Budhidarmo, R., Mace, P.D., Shirley, S., Condon, S.M., Chunduru, S.K., McKinlay, M.A., Vaux, D.L., Silke, J.*, et al.* (2011). Smac mimetics activate the E3 ligase activity of cIAP1 protein by promoting RING domain dimerization. Journal of Biological Chemistry *286*, 17015-17028.

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

Fichelson, P., and Gho, M. (2003). The glial cell undergoes apoptosis in the microchaete lineage of Drosophila. Development *130*, 123-133.

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

Freel, C.D., Richardson, D.A., Thomenius, M.J., Gan, E.C., Horn, S.R., Olson, M.R., and Kornbluth, S. (2008). Mitochondrial localization of Reaper to promote inhibitors of

apoptosis protein degradation conferred by GH3 domain-lipid interactions. J Biol Chem *283*, 367-379.

Fuchs, Y., Brown, S., Gorenc, T., Rodriguez, J., Fuchs, E., and Steller, H. (2013). Sept4/ARTS regulates stem cell apoptosis and skin regeneration. Science *341*, 286-289.

Fuentes-Prior, P., and Salvesen, G.S. (2004). The protein structures that shape caspase activity, specificity, activation and inhibition. Biochemical Journal *384*, 201-232.

Galindo, K.A., Lu, W.J., Park, J.H., and Abrams, J.M. (2009). The Bax/Bak ortholog in Drosophila, Debcl, exerts limited control over programmed cell death. Development *136*, 275-283.

Gao, Y., Han, C., Huang, H., Xin, Y., Xu, Y., Luo, L., and Yin, Z. (2010). Heat shock protein 70 together with its co-chaperone CHIP inhibits TNF-alpha induced apoptosis by promoting proteasomal degradation of apoptosis signal-regulating kinase1. Apoptosis : an international journal on programmed cell death *15*, 822-833.

Garneau, N.L., Wilusz, J., and Wilusz, C.J. (2007). The highways and byways of mRNA decay. Nature reviews Molecular cell biology *8*, 113-126.

Ge, W., Chen, Y.W., Weng, R., Lim, S.F., Buescher, M., Zhang, R., and Cohen, S.M. (2012). Overlapping functions of microRNAs in control of apoptosis during Drosophila embryogenesis. Cell Death Differ *19*, 839-846.

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

Graves, J.D., Gotoh, Y., Draves, K.E., Ambrose, D., Han, D.K.M., Wright, M., Chernoff, J., Clark, E.A., and Krebs, E.G. (1998). Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. Embo J *17*, 2224-2234.

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

Guo, J.Y., Xia, B., and White, E. (2013). Autophagy-mediated tumor promotion. Cell *155*, 1216-1219.

Gyrd-Hansen, M., and Meier, P. (2010). IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nature reviews Cancer *10*, 561-574.

Hao, Y.Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., Ohata, H., Katayama, R., Hashimoto, C., Zhang, X.D., Noda, T.*, et al.* (2004). Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. Nat Cell Biol *6*, 849-860.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell *114*, 457-467.

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

Hays, R., Wickline, L., and Cagan, R. (2002). Morgue mediates apoptosis in the Drosophila melanogaster retina by promoting degradation of DIAP1. Nat Cell Biol *4*, 425-431.

Hegde, R., Srinivasula, S.M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A.S., Fernandes-Alnemri, T.*, et al.* (2002). Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem *277*, 432-438.

Helfer, B., Boswell, B.C., Finlay, D., Cipres, A., Vuori, K., Bong Kang, T., Wallach, D., Dorfleutner, A., Lahti, J.M., Flynn, D.C.*, et al.* (2006). Caspase-8 promotes cell motility and calpain activity under nonapoptotic conditions. Cancer research *66*, 4273-4278.

Hennessy, E.J., Adam, A., Aquila, B.M., Castriotta, L.M., Cook, D., Hattersley, M., Hird, A.W., Huntington, C., Kamhi, V.M., Laing, N.M.*, et al.* (2013). Discovery of a novel class of dimeric Smac mimetics as potent IAP antagonists resulting in a clinical candidate for the treatment of cancer (AZD5582). Journal of medicinal chemistry *56*, 9897-9919.

Hillebrand, J., Barbee, S.A., and Ramaswami, M. (2007). P-body components, microRNA regulation, and synaptic plasticity. TheScientificWorldJournal *7*, 178-190.

Hillebrand, J., Pan, K., Kokaram, A., Barbee, S., Parker, R., and Ramaswami, M. (2010). The Me31B DEAD-box helicase localizes to postsynaptic foci and regulates expression of a CaMKII reporter mRNA in dendrites of Drosophila olfactory projection neurons. Frontiers in neural circuits *4*, 121.

Holley, C.L., Olson, M.R., Colon-Ramos, D.A., and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. Nat Cell Biol *4*, 439-444.

Hu, S., and Yang, X. (2003). Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. Journal of Biological Chemistry *278*, 10055-10060.

Huang, L., Mollet, S., Souquere, S., Le Roy, F., Ernoult-Lange, M., Pierron, G., Dautry, F., and Weil, D. (2011). Mitochondria associate with P-bodies and modulate microRNA-mediated RNA interference. The Journal of biological chemistry *286*, 24219-24230.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., and Wu, H. (2001). Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell *104*, 781-790.

Huh, J.R., Foe, I., Muro, I., Chen, C.H., Seol, J.H., Yoo, S.J., Guo, M., Park, J.M., and Hay, B.A. (2007). The Drosophila inhibitor of apoptosis (IAP) DIAP2 is dispensable for cell survival, required for the innate immune response to gram-negative bacterial infection, and can be negatively regulated by the reaper/hid/grim family of IAP-binding apoptosis inducers. The Journal of biological chemistry *282*, 2056-2068.

Huh, J.R., Vernooy, S.Y., Yu, H., Yan, N., Shi, Y., Guo, M., and Hay, B.A. (2003). Multiple apoptotic caspase cascades are required in nonapoptotic roles for Drosophila spermatid individualization. PLoS Biol *2*, e15.

Jana, N.R., Dikshit, P., Goswami, A., Kotliarova, S., Murata, S., Tanaka, K., and Nukina, N. (2005). Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. J Biol Chem *280*, 11635-11640.

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

Jinek, M., Eulalio, A., Lingel, A., Helms, S., Conti, E., and Izaurralde, E. (2008). The C-terminal region of Ge-1 presents conserved structural features required for P-body localization. RNA *14*, 1991-1998.

Kaczmarek, A., Vandenabeele, P., and Krysko, D.V. (2013). Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity *38*, 209-223.

Kedersha, N., Cho, M.R., Li, W., Yacono, P.W., Chen, S., Gilks, N., Golan, D.E., and Anderson, P. (2000). Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. J Cell Biol *151*, 1257-1268.

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

Kim, M., Bellini, M., and Ceman, S. (2009). Fragile X mental retardation protein FMRP binds mRNAs in the nucleus. Mol Cell Biol *29*, 214-228.

Kondo, S., Senoo-Matsuda, N., Hiromi, Y., and Miura, M. (2006). DRONC coordinates cell death and compensatory proliferation. Mol Cell Biol *26*, 7258-7268.

Kornbluth, S., and White, K. (2005). Apoptosis in Drosophila: neither fish nor fowl (nor man, nor worm). Journal of Cell Science *118*, 1779-1787.

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

Kumar, S. (2007). Caspase function in programmed cell death. Cell Death Differ *14*, 32-43.

Kuranaga, E., Kanuka, H., Tonoki, A., Takemoto, K., Tomioka, T., Kobayashi, M., Hayashi, S., and Miura, M. (2006). Drosophila IKK-related kinase regulates nonapoptotic function of caspases via degradation of IAPs. Cell *126*, 583-596.

Landais, I., Lee, H., and Lu, H. (2006). Coupling caspase cleavage and ubiquitin-proteasome-dependent degradation of SSRP1 during apoptosis. Cell Death Differ *13*, 1866-1878.

Lannan, E., Vandergaast, R., and Friesen, P.D. (2007). Baculovirus caspase inhibitors P49 and P35 block virus-induced apoptosis downstream of effector caspase DrICE activation in Drosophila melanogaster cells. Journal of virology *81*, 9319-9330.

Larisch, S., Yi, Y., Lotan, R., Kerner, H., Eimerl, S., Tony Parks, W., Gottfried, Y., Birkey Reffey, S., de Caestecker, M.P., Danielpour, D*., et al.* (2000). A novel mitochondrial septin-like protein, ARTS, mediates apoptosis dependent on its P-loop motif. Nat Cell Biol *2*, 915-921.

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

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

Lee, G., Sehgal, R., Wang, Z., Nair, S., Kikuno, K., Chen, C.H., Hay, B., and Park, J.H. (2013). Essential role of grim-led programmed cell death for the establishment of corazonin-producing peptidergic nervous system during embryogenesis and metamorphosis in Drosophila melanogaster. Biology open *2*, 283-294.

Lee, T.V., Fan, Y., Wang, S., Srivastava, M., Broemer, M., Meier, P., and Bergmann, A. (2011). Drosophila IAP1-mediated ubiquitylation controls activation of the initiator caspase DRONC independent of protein degradation. PLoS Genet *7*, e1002261.

Leulier, F., Ribeiro, P.S., Palmer, E., Tenev, T., Takahashi, K., Robertson, D., Zachariou, A., Pichaud, F., Ueda, R., and Meier, P. (2006). Systematic in vivo RNAi analysis of putative components of the Drosophila cell death machinery. Cell Death Differ *13*, 1663-1674.

Li, J., and Yuan, J. (2008). Caspases in apoptosis and beyond. Oncogene *27*, 6194-6206.

Li, X., Wang, J., and Shi, Y. (2011). Structural mechanisms of DIAP1 auto-inhibition and DIAP1-mediated inhibition of drICE. Nature communications *2*, 408.

Lin, H.V., Rogulja, A., and Cadigan, K.M. (2004). Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis. Development *131*, 2409-2418.

Linkermann, A., and Green, D.R. (2014). Necroptosis. The New England journal of medicine *370*, 455-465.

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

Luthi, A.U., and Martin, S.J. (2007). The CASBAH: a searchable database of caspase substrates. Cell Death Differ *14*, 641-650.

MacFarlane, M., Merrison, W., Bratton, S.B., and Cohen, G.M. (2002). Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. Journal of Biological Chemistry *277*, 36611-36616.

Malladi, S., Challa-Malladi, M., Fearnhead, H.O., and Bratton, S.B. (2009). The Apaf-1*procaspase-9 apoptosome complex functions as a proteolytic-based molecular timer. EMBO J *28*, 1916-1925.

Martins, L.M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N.F., Lemoine, N.R., Savopoulos, J., Gray, C.W., Creasy, C.L., Dingwall, C.*, et al.* (2002). The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem *277*, 439-444.

Means, J.C., Muro, I., and Clem, R.J. (2006). Lack of involvement of mitochondrial factors in caspase activation in a Drosophila cell-free system. Cell Death Differ *13*, 1222-1234.

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

Meinander, A., Runchel, C., Tenev, T., Chen, L., Kim, C.H., Ribeiro, P.S., Broemer, M., Leulier, F., Zvelebil, M., Silverman, N.*, et al.* (2012). Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling. The EMBO journal *31*, 2770-2783.

Minshall, N., and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. Nucleic acids research *32*, 1325-1334.

Miura, M., Chen, X.D., Allen, M.R., Bi, Y., Gronthos, S., Seo, B.M., Lakhani, S., Flavell, R.A., Feng, X.H., Robey, P.G.*, et al.* (2004). A crucial role of caspase-3 in osteogenic differentiation of bone marrow stromal stem cells. The Journal of clinical investigation *114*, 1704-1713.

Muro, I., Berry, D.L., Huh, J.R., Chen, C.H., Huang, H., Yoo, S.J., Guo, M., Baehrecke, E.H., and Hay, B.A. (2006). The Drosophila caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process. Development *133*, 3305-3315.

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

Muro, I., Monser, K., and Clem, R.J. (2004). Mechanism of Dronc activation in Drosophila cells. J Cell Sci *117*, 5035-5041.

Naito, M., Nagashima, K., Mashima, T., and Tsuruo, T. (1997). Phosphatidylserine externalization is a downstream event of interleukin-1 beta-converting enzyme family protease activation during apoptosis. Blood *89*, 2060-2066.

Nakagawa, Y., Morikawa, H., Hirata, I., Shiozaki, M., Matsumoto, A., Maemura, K., Nishikawa, T., Niki, M., Tanigawa, N., Ikegami, M.*, et al.* (1999). Overexpression of rck/p54, a DEAD box protein, in human colorectal tumours. British journal of cancer *80*, 914-917.

Nakamura, A., Amikura, R., Hanyu, K., and Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development *128*, 3233-3242.

Navarro, R.E., Shim, E.Y., Kohara, Y., Singson, A., and Blackwell, T.K. (2001). cgh-1, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in C. elegans. Development *128*, 3221-3232.

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

Olson, M.R., Holley, C.L., Gan, E.C., Colon-Ramos, D.A., Kaplan, B., and Kornbluth, S. (2003a). A GH3-like domain in reaper is required for mitochondrial localization and induction of IAP degradation. J Biol Chem *278*, 44758-44768.

Olson, M.R., Holley, C.L., Yoo, S.J., Huh, J.R., Hay, B.A., and Kornbluth, S. (2003b). Reaper is regulated by IAP-mediated ubiquitination. Journal of Biological Chemistry *278*, 4028-4034.

Paquette, N., Broemer, M., Aggarwal, K., Chen, L., Husson, M., Erturk-Hasdemir, D., Reichhart, J.M., Meier, P., and Silverman, N. (2010). Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for Drosophila NF-kappaB signaling. Mol Cell *37*, 172-182.

Parker, R., and Sheth, U. (2007). P bodies and the control of mRNA translation and degradation. Mol Cell *25*, 635-646.

Plesca, D., Mazumder, S., Gama, V., Matsuyama, S., and Almasan, A. (2008). A C-terminal fragment of cyclin E, generated by caspase-mediated cleavage, is degraded in the absence of a recognizable phosphodegron. Journal of Biological Chemistry *283*, 30796-30803.

Pluta, A., Wrzesien-Kus, A., Cebula-Obrzut, B., Wolska, A., Szmigielska-Kaplon, A., Czemerska, M., Pluta, P., Robak, T., Smolewski, P., and Wierzbowska, A. (2010). Influence of high expression of Smac/DIABLO protein on the clinical outcome in acute myeloid leukemia patients. Leukemia research *34*, 1308-1313.

Pop, C., and Salvesen, G.S. (2009). Human caspases: activation, specificity, and regulation. J Biol Chem *284*, 21777-21781.

Riedl, S.J., Renatus, M., Schwarzenbacher, R., Zhou, Q., Sun, C.H., Fesik, S.W., Liddington, R.C., and Salvesen, G.S. (2001). Structural basis for the inhibition of caspase-3 by XIAP. Cell *104*, 791-800.

Rovani, M.K., Brachmann, C.B., Ramsay, G., and Katzen, A.L. (2012). The dREAM/Myb-MuvB complex and Grim are key regulators of the programmed death of neural precursor cells at the Drosophila posterior wing margin. Dev Biol *372*, 88-102.

Ryoo, H.D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. (2002). Regulation of Drosophila IAP1 degradation and apoptosis by reaper and ubcD1. Nat Cell Biol *4*, 432-438.

Salvesen, G.S., and Duckett, C.S. (2002). IAP proteins: Blocking the road to death's door. Nat Rev Mol Cell Biol *3*, 401-410.

Samuel, T., Welsh, K., Lober, T., Togo, S.H., Zapata, J.M., and Reed, J.C. (2006). Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. J Biol Chem *281*, 1080-1090.

Sandu, C., Ryoo, H.D., and Steller, H. (2010). Drosophila IAP antagonists form multimeric complexes to promote cell death. The Journal of Cell Biology *190*, 1039-1052.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H., and Wanker, E.E. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell *90*, 549-558.

Scott, F.L., Denault, J.B., Riedl, S.J., Shin, H., Renatus, M., and Salvesen, G.S. (2005). XIAP inhibits caspase-3 and -7 using two binding sites: evolutionarily conserved mechanism of IAPs. EMBO J *24*, 645-655.

Sears, N., Sen, G.C., Stark, G.R., and Chattopadhyay, S. (2011). Caspase-8-mediated cleavage inhibits IRF-3 protein by facilitating its proteasome-mediated degradation. Journal of Biological Chemistry *286*, 33037-33044.

Semple, J.I., Smits, V.A.J., Fernaud, J.R., Mamely, I., and Freire, R. (2007). Cleavage and degradation of Claspin during apoptosis by caspases and the proteasome. Cell Death Differ *14*, 1433-1442.

Shabek, N., Herman-Bachinsky, Y., Buchsbaum, S., Lewinson, O., Haj-Yahya, M., Hejjaoui, M., Lashuel, H.A., Sommer, T., Brik, A., and Ciechanover, A. (2012). The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. Mol Cell *48*, 87-97.

Shao, J., and Diamond, M.I. (2007). Polyglutamine diseases: emerging concepts in pathogenesis and therapy. Human molecular genetics *16 Spec No. 2*, R115-123.

She, M., Decker, C.J., Svergun, D.I., Round, A., Chen, N., Muhlrad, D., Parker, R., and Song, H. (2008). Structural basis of dcp2 recognition and activation by dcp1. Mol Cell *29*, 337-349.

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

Shukla, A., and Tapadia, M.G. (2011). Differential localization and processing of apoptotic proteins in Malpighian tubules of Drosophila during metamorphosis. European Journal of Cell Biology *90*, 72-80.

Silke, J., Kratina, T., Chu, D., Ekert, P.G., Day, C.L., Pakusch, M., Huang, D.C.S., and Vaux, D.L. (2005). Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance. Proceedings of the National Academy of Sciences of the United States of America *102*, 16182-16187.

Silke, J., Kratina, T., Ekert, P.G., Pakusch, M., and Vaux, D.L. (2004). Unlike Diablo/smac, Grim promotes global ubiquitination and specific degradation of X chromosome-linked inhibitor of apoptosis (XIAP) and neither cause apoptosis. J Biol Chem *279*, 4313-4321.

Smillie, D.A., and Sommerville, J. (2002). RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. J Cell Sci *115*, 395-407.

Smith, M.C., Scaglione, K.M., Assimon, V.A., Patury, S., Thompson, A.D., Dickey, C.A., Southworth, D.R., Paulson, H.L., Gestwicki, J.E., and Zuiderweg, E.R. (2013). The E3 ubiquitin ligase CHIP and the molecular chaperone Hsc70 form a dynamic, tethered complex. Biochemistry *52*, 5354-5364.

Snipas, S.J., Drag, M., Stennicke, H.R., and Salvesen, G.S. (2008). Activation mechanism and substrate specificity of the Drosophila initiator caspase DRONC. Cell Death Differ *15*, 938-945.

Srinivasula, S.M., Datta, P., Kobayashi, M., Wu, J.W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y.*, et al.* (2002). sickle, a novel Drosophila death gene in the reaper/hid/grim region, encodes an IAP-inhibitory protein. Curr Biol *12*, 125-130.

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

Stennicke, H.R., Renatus, M., Meldal, M., and Salvesen, G.S. (2000). Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. The Biochemical journal *350 Pt 2*, 563-568.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K., and Takahashi, R. (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell *8*, 613-621.

Tait, S.W., Werner, A.B., de Vries, E., and Borst, J. (2004). Mechanism of action of Drosophila Reaper in mammalian cells: Reaper globally inhibits protein synthesis and induces apoptosis independent of mitochondrial permeability. Cell Death Differ *11*, 800-811.

Tan, Y., Yamada-Mabuchi, M., Arya, R., St Pierre, S., Tang, W., Tosa, M., Brachmann, C., and White, K. (2011). Coordinated expression of cell death genes regulates neuroblast apoptosis. Development *138*, 2197-2206.

Tapadia, M.G., and Gautam, N.K. (2011). Non-apoptotic function of apoptotic proteins in the development of Malpighian tubules of Drosophila melanogaster. J Biosci *36*, 531-544.

Teixeira, D., Sheth, U., Valencia-Sanchez, M.A., Brengues, M., and Parker, R. (2005). Processing bodies require RNA for assembly and contain nontranslating mRNAs. RNA *11*, 371-382.

Tenev, T., Zachariou, A., Wilson, R., Ditzel, M., and Meier, P. (2005). IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. Nat Cell Biol *7*, 70-+.

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

Thomenius, M., Freel, C.D., Horn, S., Krieser, R., Abdelwahid, E., Cannon, R., Balasundaram, S., White, K., and Kornbluth, S. (2011). Mitochondrial fusion is regulated by Reaper to modulate Drosophila programmed cell death. Cell Death Differ *18*, 1640-1650.

Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M. (2000). Recognition of the polyubiquitin proteolytic signal. Embo J *19*, 94-102.

Tikhomirov, O., and Carpenter, G. (2001). Caspase-dependent cleavage of ErbB-2 by geldanamycin and staurosporin. Journal of Biological Chemistry *276*, 33675-33680.

Tritschler, F., Braun, J.E., Motz, C., Igreja, C., Haas, G., Truffault, V., Izaurralde, E., and Weichenrieder, O. (2009). DCP1 forms asymmetric trimers to assemble into active mRNA decapping complexes in metazoa. Proc Natl Acad Sci U S A *106*, 21591-21596.

Tritschler, F., Eulalio, A., Helms, S., Schmidt, S., Coles, M., Weichenrieder, O., Izaurralde, E., and Truffault, V. (2008). Similar modes of interaction enable Trailer Hitch and EDC3 to associate with DCP1 and Me31B in distinct protein complexes. Mol Cell Biol *28*, 6695-6708.

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

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

Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. Journal of immunological methods *184*, 39-51.

Waldhuber, M., Emoto, K., and Petritsch, C. (2005). The Drosophila caspase DRONC is required for metamorphosis and cell death in response to irradiation and developmental signals. Mechanisms of development *122*, 914-927.

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

White, E. (2012). Deconvoluting the context-dependent role for autophagy in cancer. Nature reviews Cancer *12*, 401-410.

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

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

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

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

Wright, C.W., and Clem, R.J. (2002). Sequence requirements for Hid binding and apoptosis regulation in the baculovirus inhibitor of apoptosis Op-IAP. Hid binds Op-IAP in a manner similar to Smac binding of XIAP. J Biol Chem *277*, 2454-2462.

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

Wu, J.N., Nguyen, N., Aghazarian, M., Tan, Y., Sevrioukov, E.A., Mabuchi, M., Tang, W., Monserrate, J.P., White, K., and Brachmann, C.B. (2010). grim promotes programmed cell death of Drosophila microchaete glial cells. Mechanisms of Development *127*, 407-417.

Wu, J.W., Cocina, A.E., Chai, J.J., Hay, B.A., and Shi, Y.G. (2001). Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. Mol Cell *8*, 95-104.

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

Yeh, T.C., and Bratton, S.B. (2013). Caspase-dependent regulation of the ubiquitin-proteasome system through direct substrate targeting. Proceedings of the National Academy of Sciences of the United States of America *110*, 14284-14289.

Yeh, T.C., and Bratton, S.B. (2014). DrICE resurrects Grim to antagonize DIAP1. Cell cycle *13*.

Yokokura, T., Dresnek, D., Huseinovic, N., Lisi, S., Abdelwahid, E., Bangs, P., and White, K. (2004). Dissection of DIAP1 functional domains via a mutant replacement strategy. Journal of Biological Chemistry *279*, 52603-52612.

Yoo, S.J. (2005). Grim stimulates Diap poly-ubiquitination by binding to UbcD1. Mol Cells *20*, 446-451.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., and Hay, B.A. (2002). Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat Cell Biol *4*, 416-424.

Yuan, S., Yu, X., Topf, M., Dorstyn, L., Kumar, S., Ludtke, S.J., and Akey, C.W. (2011). Structure of the Drosophila apoptosome at 6.9 a resolution. Structure *19*, 128-140.

Zachariou, A., Tenev, T., Goyal, L., Agapite, J., Steller, H., and Meier, P. (2003). IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. EMBO J *22*, 6642-6652.

Zhuang, M., Guan, S., Wang, H., Burlingame, A.L., and Wells, J.A. (2013). Substrates of IAP ubiquitin ligases identified with a designed orthogonal E3 ligase, the NEDDylator. Mol Cell *49*, 273-282.

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

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