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Caspase-Dependent Cell Death in *Drosophila*

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Annu. Rev. Cell Dev. Biol. 2006. 22:623–50

First published online as a Review in Advance on July 14, 2006

The *Annual Review of Cell and Developmental Biology* is online at http://cellbio.annualreviews.org

This article's doi: 10.1146/annurev.cellbio.21.012804.093845

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1081-0706/06/1110-0623$20.00

Key Words

DIAP1, apoptosis, neurodegeneration, spermatogenesis, DIAP2, innate immunity, proliferation

Abstract

Cell death plays many roles during development, in the adult, and in the genesis of many pathological states. Much of this death is apoptotic in nature and requires the activity of members of the caspase family of proteases. It is now possible uniquely in *Drosophila* to carry out genetic screens for genes that determine the fate—life or death—of any population of cells during development and adulthood. This, in conjunction with the ability to obtain biochemical quantities of material, has made *Drosophila* a useful organism for exploring the mechanisms by which apoptosis is carried out and regulated. This review summarizes our knowledge of caspase-dependent cell death in *Drosophila* and compares that knowledge with what is known in worms and mammals. We also discuss the significance of recent work showing that a number of key cell death activators also play nonapoptotic roles. We highlight opportunities and outstanding questions along the way.

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INTRODUCTION

Apoptotic cell death is ubiquitous in animals, in which it serves to remove cells during development and adulthood that are no longer needed, damaged, or infected (Baehrecke 2002, Benedict et al. 2002, Green & Evan 2002, Opferman & Korsmeyer 2003). Members of a family of proteases known as caspases comprise the core of the apoptosis cell death machine (Degterev et al. 2003). Caspases are expressed ubiquitously and synthesized as relatively inactive zymogens. As discussed below, several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. Interestingly, however, different organisms seem to emphasize distinct points of control.

In *Caenorhabditis elegans*, the caspase CED-3 is required for apoptotic cell death (reviewed in Conradt & Xue 2005). CED-3 and its activator, the adaptor protein CED-4, are present ubiquitously. CED-4-dependent activation of CED-3 is mediated by interactions between the two proteins involving the N-terminal CARD domains present in both proteins. CED-4's ability to activate CED-3 is thought to be constitutive, and no inhibitors of

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activated CED-3 have been identified. However, most cells are protected from death by the expression of CED-9, an antiapoptotic, multidomain Bcl-2 family protein that sequesters CED-4 at mitochondria and prevents CED-4 oligomerization required to facilitate CED-3 activation. In many (but not all) cells chosen to die, a small proapoptotic protein EGL-1 (a member of the BH3-domain-only Bcl-2 family) that binds to CED-9 is expressed. EGL-1 expression triggers a conformational change in CED-9 that results in the release of CED-4 from the inhibitory CED-4–CED-9 complex, thereby allowing CED-4 to oligomerize further and promote CED-3 activation (Yan et al. 2004a, 2005; Yan & Shi 2005). Thus, in this system, the decision to activate caspase-dependent cell death is made at an upstream level, through the inhibition of CED-9, an inhibitor of caspase activation (see **Figure 1**).

In contrast, in mammals, the primary decision to activate caspase-dependent death is usually made at the level of positive death signals that drive the activation of long prodomain initiator caspases (**Figure 1**). These promote apoptosis by cleaving and activating short prodomain effector caspases that target other cellular proteins for cleavage and inactivation or activation. Active caspases in mammals and flies, but not *C. elegans*, are negatively regulated by members of the inhibitor of apoptosis protein (IAP) family (Salvesen & Duckett 2002, Vaux & Silke 2005, Yan & Shi 2005).

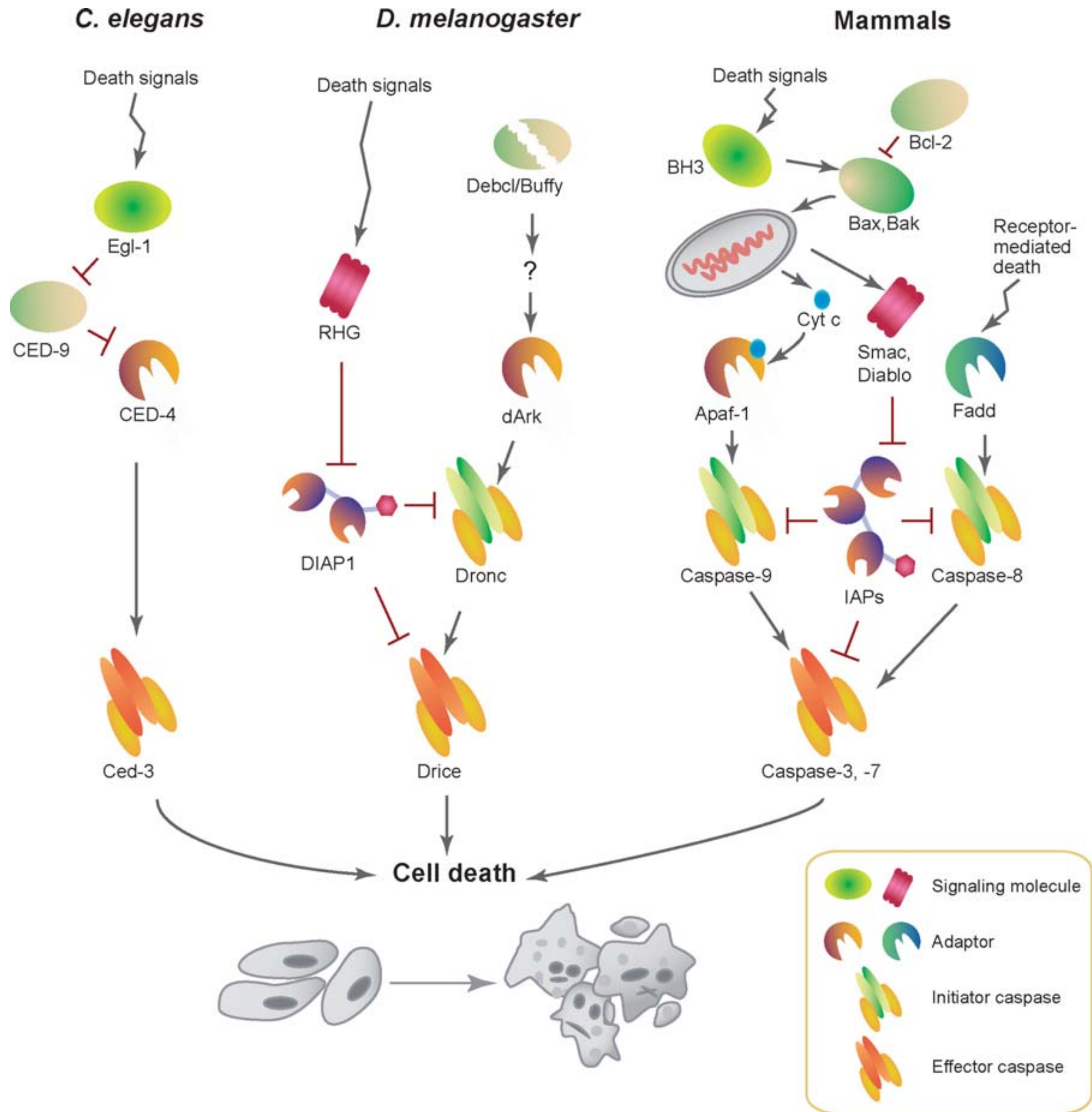
In contrast to the systems mentioned above, in which caspase activation serves as the primary point of control, in *Drosophila* many cells experience chronic activation of the initiator caspase Dronc, the fly equivalent to mammalian caspase-9. Dronc activation is mediated by the adaptor Ark, the fly homolog of CED-4 in worms, and Apaf-1 in mammals. Cells survive this continuous death stimulus because they express DIAP1, an IAP family caspase inhibitor. In this system cell death is induced by the regulated expression of proteins that disrupt DIAP1-caspase interactions, thereby unleashing a cascade of apoptosis-inducing caspase activity (**Figure 1**) (reviewed in Hay et al. 2004). In the sections below we describe in more detail caspase-dependent death and the ways in which it is regulated in *Drosophila*. We also highlight unanswered questions (and thus future opportunities) and exceptions that point to the existence of alternative pathways.

THE CORE OF A *DROSOPHILA* CASPASE-DEPENDENT CELL DEATH MACHINE: Ark, Dronc, AND Drice

The *Drosophila* genome encodes seven caspases. Dronc (Dorstyn et al. 1999a), Strica (Dream) (Vernooy et al. 2000, Doumanis et al. 2001), and Dredd (Chen et al. 1998) contain long N-terminal prodomains, suggesting that they function as initiator caspases. Drice (Fraser & Evan 1997), Dcp-1 (Song et al. 1997), Decay (Dorstyn et al. 1999b), and Damm (Daydream) (Vernooy et al. 2000, Harvey et al. 2001) contain short prodomains characteristic of caspases activated through cleavage. Dredd was initially isolated as a potential inducer of apoptosis functioning downstream of the death activator Reaper (Rpr) (Chen et al. 1998). Roles for Dredd in apoptosis have not been excluded, but Dredd is now primarily recognized for its essential role in a nonapoptotic process, the activation of the innate immune response following infection by gram-negative bacteria (Hultmark 2003). Strica contains a novel, serine- and threonine-rich prodomain, and ectopic expression of this caspase can induce cell death (as can overexpression of many proteases) (Doumanis et al. 2001, Xu et al. 2003). However, proteins that might mediate Strica activation have not been identified, and nothing is known about the biological processes in which Strica participates. In contrast, abundant evidence demonstrates a critical role for Dronc as an apical cell death caspase.

Ark-Dependent Activation of Dronc Initiates Many, but Not All Cell Deaths

Dronc contains an N-terminal CARD domain, as does the fly CED-4/Apaf-1 homolog known as Ark (Hac-1, Dapaf-1) (Kanuka et al. 1999, Rodriguez et al. 1999, Zhou et al. 1999). Ark and Dronc form high-molecular-weight complexes (Quinn et al. 2000, Dorstyn et al. 2002, Yu et al. 2006), and at least in *Drosophila* S2 cells, Dark is required for Dronc autoprocessing, which is required for Dronc activation (Muro et al. 2002, 2004; Yan et al. 2006). Dronc has unique substrate specificity among caspases, cleaving after aspartate, similar to other caspases, and after glutamate residues (Hawkins et al. 2000b). Dronc autoprocessing occurs after glutamate, and cleavage at this site



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is required for Dronc activity (Hawkins et al. 2000b, Muro et al. 2004, Yan et al. 2006).

Monomeric caspase-9 is recruited to the mammalian apoptosome through its CARD domain. This complex functions as a holoenzyme, in which monomeric caspase-9 is the catalytic subunit and Apaf-1 is its catalytic regulator (Rodriguez & Lazebnik 1999). Dronc activation proceeds through a quite different mechanism. Dronc undergoes autocleavage at E352 (through an unknown mechanism, but presumably following its recruitment into the apoptosome through its CARD domain), and this triggers a transition from an inactive monomer to a catalytically active dimer in which adjacent monomers mutually stabilize each other's active sites (Yan et al. 2006). Once activated by cleavage following E352, Dronc can cleave itself following E143 (Yan et al. 2006). The biology of this cleavage event may be interesting. First, cleavage following E143 removes the prodomain from the catalytic core domain, which remains active. Cleavage is thus expected to liberate a catalytically active version of Dronc from the apoptosome. It is not known if the activated apoptosome has a specific subcellular localization. If it does, cleavage at this site would presumably allow the diffusion (or transport) of active Dronc to new locations. Cleavage following E145 also removes Dronc sequences needed to mediate binding and inhibition by DIAP1 (Chai et al. 2003). These events may be important, as Ark and Dronc have been implicated in several nonapoptotic processes (see below) (Geisbrecht & Montell 2004; Huh et al. 2004a,b; Arama et al. 2005).

An unanswered question in these contexts is how cells with active Dronc avoid cell death. The sequestration of active, but inhibitable, Dronc may play a role. For example, active Dronc bound to the apoptosome may be less able than free, prodomainless active Dronc to access apoptosis-inducing targets and/or be better able to access targets that mediate nonapoptotic functions. In either case the prodomain-containing form would still be subject to DIAP1 inhibition. Therefore, in such a model, anything that stimulated cleavage following E145 would promote apoptosis: by allowing the free movement of active Dronc that cannot be inhibited by DIAP1. It will be interesting to see if this cleavage event is in fact regulated.

Multiple lines of evidence demonstrate that Ark and Dronc are important for bringing

Figure 1

Comparison of caspase-dependent cell death in *C. elegans*, *Drosophila*, and mammals. In *C. elegans* the adaptor CED-4 promotes the activation of the caspase CED-3. CED-9, an antiapoptotic multidomain Bcl-2 family member, inhibits CED-4 activity in cells that normally live. Death is induced through the expression of EGL-1, a BH3-domain-only proapoptotic Bcl-2 family member that disrupts interactions between CED-9 and CED-4. In *D. melanogaster* the adaptor Ark (homologous to CED-4 and Apaf-1) promotes the activation of the initiator caspase Dronc. Pro- and antiapoptotic multidomain Bcl-2 family members Debcl and Buffy may regulate this activation, but this is highly speculative at this point (thus the *question mark* associated with the *arrow*). The inhibitor of apoptosis protein (IAP) DIAP1 inhibits Dronc and effector caspases activated by Dronc such as Drice. DIAP1-binding proteins such as Rpr, Hid, Grim, Sickle, and Jafrac-2 (RHG) promote death in part by disrupting DIAP1's ability to inhibit caspase activity. In mammals, Apaf-1-dependent activation of caspase-9 (functionally homologous to Dronc) is highly regulated by Bcl-2 family proteins. Antiapoptotic multidomain proteins are represented by Bcl-2, and proapoptotic multidomain proteins by Bax and Bak. Many different death stimuli promote the expression or activation of BH3-domain-only family members, which facilitate Bax- and Bak-dependent release of proapoptotic, mitochondrially localized proteins, including cytochrome *c*, which promotes Apaf-1 activity, and the IAP-binding protein Smac/Diablo, which disrupts IAP-anticaspase activity (Jiang & Wang 2004). In a separate pathway, ligand-bound death receptors recruit adaptors such as Fadd, which then recruit and activate apical caspases such as caspase-8 (Lavrik et al. 2005). In both pathways apical caspase activation leads to cleavage and the activation of downstream caspases such as caspase-3 and caspase-7. In both pathways IAPs inhibit active caspases.

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about cell death. Mutants of *ark* show decreased cell death in several different contexts (Kanuka et al. 1999; Rodriguez et al. 1999, 2002; Zhou et al. 1999; Akdemir et al. 2006; Mills et al. 2006; Srivastava et al. 2006), and RNA interference (RNAi) of *ark* blocks some, but not all, caspase-dependent cell deaths (Muro et al. 2002, 2004; Zimmermann et al. 2002; Kiessling & Green 2006; Leulier et al. 2006). The expression of dominant-negative versions of Dronc blocks caspase-dependent cell death in the eye (Hawkins et al. 2000b, Meier et al. 2000), as does RNAi of *dronc* in the embryo (Quinn et al. 2000), in the eye (Leulier et al. 2006), or in S2 cells (Igaki et al. 2002; Muro et al. 2002, 2004; Kiessling & Green 2006). Most recently, multiple groups have shown that animals lacking *dronc* have reduced cell death in a number of different contexts. These contexts include the development of the embryo, eye, wing, central nervous system, and larval salivary gland and in response to multiple stresses such as RNA or protein synthesis inhibition, or DNA damage (Chew et al. 2004, Daish et al. 2004, Waldhuber et al. 2005, Xu et al. 2005).

Drice Is an Important Cell Death Effector Caspase

What are important Dronc targets? Expression of the baculovirus caspase inhibitor p35 inhibits Dronc-dependent cell death, but it does not inhibit Dronc (Hay et al. 1994, Hawkins et al. 2000b, Meier et al. 2000, Martin & Baehrecke 2004). These observations suggest that p35-sensitive effector caspases, which in at least some cases are activated by Dronc, are critical for bringing about cell death. The only known Dronc substrates are Dcp-1, Drice (Hawkins et al. 2000b, Meier et al. 2000), and DIAP1 (Yan et al. 2004b, Muro et al. 2005). Drice and Dcp-1 are highly homologous to each other and are the most homologous among the *Drosophila* caspases to several important mammalian death effector caspases, caspase-3 and caspase-7. In addition, they are p35 sensitive (Hawkins et al. 1999, Wang et al. 1999). Several observations indicate that Drice is an important effector caspase. First, the depletion of Drice from S2 cells inhibits apoptotic events in response to a variety of stimuli now known to be Dronc dependent (Fraser et al. 1997; Muro et al. 2002, 2004). In addition, antibodies that recognize the Dronc-cleaved, and therefore activated, version of Drice (anti-active Drice) label dying cells during development as well as cells exposed to other apoptotic stimuli (Yoo et al. 2002, Yu et al. 2002, Muro et al. 2006, Xu et al. 2006). Finally, as with *dronc* mutants, animals that lack *drice* are mostly pupal lethal and have reduced cell death in the embryonic nervous system, pupal retina, and adult wing and in response to stresses such as X-irradiation or the inhibition of protein synthesis (Muro et al. 2006, Xu et al. 2006). In contrast, *dcp-1*-null mutants are quite healthy as homozygotes. They do show defects in starvation-induced cell death during oogenesis but appear otherwise normal (Laundrie et al. 2003). Despite the paucity of clear cell death phenotypes in animals lacking *dcp-1*, Dcp-1 probably plays a partly redundant role with Drice and/or is able to compensate for its loss. *drice*, *dcp-1* double mutants showing phenotypes that are in some cases more severe than those of animals lacking only *drice* support this conclusion (Muro et al. 2006, Xu et al. 2006).

Evidence for Alternative Cell Death Pathways

Together the above results are consistent with a linear model in which many cell deaths require Ark-dependent activation of Dronc, which subsequently activates Drice. Activated Drice then cleaves cellular proteins that promote cell death in various ways. However, the observations in hand (largely of the form "the cell lives or dies in a particular mutant background") do not allow us to conclude that Ark only works through Dronc or that Dronc is only activated by Ark. They also do not address the question of whether Drice is the only effector of Dronc or whether Drice only

works through Dronc. In addition, a number of apoptotic cell deaths are Dronc and Drice independent. Thus, some cell death still occurs in embryos lacking maternal and zygotic Dronc (Chew et al. 2004; Xu et al. 2005, 2006) or Drice (Muro et al. 2006, Xu et al. 2006), and death of the larval midgut occurs on schedule (Daish et al. 2004). Most remarkably, some animals lacking *dronc* or *drice* survive to adulthood. This last result is particularly striking because massive amounts of cell death take place during metamorphosis as larval tissues are eliminated and replaced by imaginal disc-derived structures that will make up the adult. That at least some of these deaths utilize other caspase-dependent pathways comes from the following finding: Although salivary gland death occurs on schedule in animals that lack *drice* (Muro et al. 2006), it is inhibited by the expression of the baculovirus caspase inhibitor p35 (Jiang et al. 1997, Lee & Baehrecke 2001, Martin & Baehrecke 2004). Perhaps Dcp-1 works with Drice to bring about these deaths. Alternatively, and/or in addition, other caspases that remain relatively uncharacterized as cell death regulators (Strica, Damm, Decay) may be important.

As the following examples illustrate, caspase-independent pathways to cell death are also likely to be important in *Drosophila*. First, female germline nurse cells deposit their contents into the growing oocyte. Shortly thereafter, they die. These deaths do not require known upstream activators of Dronc (*reaper*, *head involution defective*, *grim*), *dcp-1*, *drice*, or *dronc*; fail to show evidence of caspase activation in reporter assays; and are insensitive to p35 or DIAP1 expression, suggesting that they may be caspase independent (Foley & Cooley 1998; Peterson et al. 2003; McCall 2004; Muro et al. 2006; Xu et al. 2005, 2006; Mazzalupo & Cooley 2006). Second, embryos in which effector caspase activity has been inhibited through p35 expression, or in which several essential upstream activators of Dronc have been deleted, can still be phagocytosed and presumably eliminated (Mergliano & Minden 2003). Although the involvement of Dronc- and p35-independent caspases cannot be excluded in these experiments, caspase-independent pathways may also play a role.

Third, during metamorphosis, massive amounts of larval tissue are eliminated and replaced by cells derived from imaginal discs. Caspase-dependent pathways clearly participate in these events. However, morphologically these deaths appear autophagic rather than apoptotic. In addition, the results of transcriptional profiling experiments indicate that tissues undergoing autophagic death express a distinct set of genes from cells undergoing classic apoptosis (Lee et al. 2003, Gorski et al. 2003). Autophagy is an ancient, conserved mechanism for degrading components of the cytoplasm. It can serve as a survival mechanism under nutrient-limiting conditions and has also been suggested to define a mechanism for bringing about cell death. Pathways that mediate autophagy appear to be activated, at least in part, in parallel with those for apoptosis in *Drosophila*, but the relationship between these two processes remains largely unexplored (Baehrecke 2005). Finally, dominant and recessive neurodegenerative diseases have been modeled in *Drosophila*. In several of these, death appears to be caspase independent, but little else is known about the effectors at work (cf. Jackson et al. 1998, Hsu et al. 2004).

DIAP1 IS AN ESSENTIAL INHIBITOR OF CASPASE-DEPENDENT CELL DEATH

IAPs were first discovered by Miller and colleagues in baculoviruses as a novel family of cell death inhibitors (Crook et al. 1993, Birnbaum et al. 1994, Clem & Miller 1994). Homologous proteins that also function as cell death inhibitors were subsequently identified in *Drosophila* (Hay et al. 1995, Vernooy et al. 2002) and mammals (reviewed in Hay 2000, Salvesen & Duckett 2002). IAP family proteins are defined by the presence of

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one or more repeats of an approximately 70-amino-acid motif known as a baculovirus IAP repeat (BIR). This motif mediates interactions with multiple death activators and plays an essential role in the ability of these proteins to inhibit cell death. However, not all proteins that contain BIRs are death inhibitors (Salvesen & Duckett 2002). In particular, the BIR-containing proteins in *C. elegans* (and their counterparts in other organisms) regulate cytokinesis rather than cell death. Many death-inhibiting IAPs also contain a C-terminal RING finger domain. This contributes to IAPs' ability to inhibit cell death in some contexts and promote death in others. This RING can act as an E3 ubiquitin ligase, recruiting ubiquitin-loaded E2 and catalyzing the transfer of ubiquitin to target proteins, including themselves (Vaux & Silke 2005). The *Drosophila* genome encodes three BIR-containing proteins shown to function as cell death inhibitors, DIAP1 (Hay et al. 1995), DIAP2 (Hay et al. 1995, Duckett et al. 1996, Liston et al. 1996, Uren et al. 1996), and dBruce (Vernooy et al. 2002). The most is known about the function of DIAP1, the focus of this section.

DIAP1 Is Required to Inhibit Ark-, Dronc-, and Drice-Dependent Cell Death

DIAP1, the product of the *thread* (*th*) locus (hereafter referred to as DIAP1), was identified in the first dominant modifier screen for cell death suppressors in the fly (Hay et al. 1995). In brief, heterozygous loss-of-function mutations in *diap1* were identified as enhancers of a small eye phenotype induced by eye-specific expression of the upstream cell death activators *rpr* or *head involution defective* (*hid*) (discussed further below). Overexpression of *diap1* also blocked cell death in multiple contexts. Most importantly, the loss of *diap1* in a number of different contexts results in cell death. These contexts include clones of tissue in the germline or eye (Hay et al. 1995), homozygous mutant embryos (Wang et al. 1999, Goyal et al. 2000, Lisi et al. 2000), RNAi in cell culture (Igaki et al. 2002, Muro et al. 2002, Zimmermann et al. 2002, Yokokura et al. 2004, Kiessling & Green 2006), and RNAi in larvae (Yin & Thummel 2004) or the developing eye (Leulier et al. 2006, Muro et al. 2006). Thus, DIAP1 is an essential inhibitor of cell death in the fly.

In vivo evidence that DIAP1's primary prosurvival function is to inhibit caspase function comes from multiple observations in the fly (Rodriguez et al. 2002; Yoo et al. 2002; Huh et al. 2004a; Xu et al. 2005, 2006; Leulier et al. 2006; Muro et al. 2006) and in cell culture (Igaki et al. 2002; Muro et al. 2002, 2004; Zimmerman et al. 2002; Kiessling & Green 2006), demonstrating that cell death caused by the loss of *diap1* can be suppressed or eliminated by decreasing the levels of *ark*, *dronc*, or *drice* or by the expression of the caspase inhibitor p35. In addition, DIAP1 overexpression blocks death induced by the expression of activated versions of Drice, Dcp-1 (Hawkins et al. 1999, Wang et al. 1999), Dronc (Hawkins et al. 2000b, Meier et al. 2000), and Strica (Doumanis et al. 2001). Importantly, when cells are rescued from death due to the loss of *diap1*, where examined, they develop normally. Examples include the ovary (a partial rescue) (Rodriguez et al. 2002, Xu et al. 2005), wing (Huh et al. 2004a), and eye (Leulier et al. 2006, Muro et al. 2006). These observations are significant because they suggest it is not the case (most of the time) that cells that have lost *diap1* undergo caspase-dependent cell death because they lack the ability to differentiate or are no longer able to carry out some other basic cellular process.

DIAP1 Inhibits Caspase Activity Through Several Mechanisms

Several observations demonstrate that DIAP1 regulates caspase activity directly. First, DIAP1 expression in yeast blocks cell death induced by the expression of activated versions of Drice, Dcp-1, and Dronc (Hawkins et al. 1999, 2000b; Wang et al. 1999;

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Meier et al. 2000). The yeast expression system, a eukaryotic but heterologous system unlikely to contain other counterparts of core *Drosophila* cell death regulators, provides a useful background for the screening of mutants in, and regulators of, DIAP1-caspase interactions, as well as for caspase substrates (Hawkins et al. 1999, 2000a; Wang et al. 1999). These experiments provided the first evidence that DIAP1, alone or in combination with other cellular components conserved throughout eukaryotic evolution, could block caspase activity. However, the studies do not explain mechanistically how DIAP1 inhibits caspase activity.

Work from a number of labs has shown that DIAP1 binds directly to Dcp-1 and Drice (Kaiser et al. 1998, Wang et al. 1999) and prevents them from cleaving substrates (Hawkins et al. 1999, Wang et al. 1999, Zachariou et al. 2003, Yan et al. 2004b, Tenev et al. 2005). Yet exactly how DIAP1 inhibits their function remains to be determined. Interestingly, active versions of these proteases are only able to bind DIAP1 following cleavage (perhaps by Dronc) at sites located near their N termini that expose a DIAP1-BIR1-binding motif necessary for a high-affinity interaction with DIAP1 (Tenev et al. 2005). Other observations show that Drice and Dcp-1 cleave DIAP1 following D20 (Ditzel et al. 2003, Yan et al. 2004b, Yokokura et al. 2004). At least in vitro, this cleavage is necessary to expose the caspase-binding motif in BIR1 (Yan et al. 2004b). Cleavage of DIAP1 at D20 has also been proposed to stimulate DIAP1 degradation through the recruitment of the N-end rule ubiquitination machinery to the new DIAP1 N terminus (Ditzel et al. 2003). Others suggest that, although cleavage of DIAP1 stimulates its degradation, this is mediated through the activation of DIAP1's RING-dependent autoubiquitination activity (Yokokura et al. 2004). In any case, the above observations support the argument that in the absence of an activating cleavage event, effector caspases and DIAP1 are invisible to each other. Evidence that the inhibition of effector caspase activity is critical for DIAP1's prosurvival function comes from the observation that the inhibition of *drice* expression in cell culture and in the fly eye inhibits death due to the loss of *diap1* (Muro et al. 2002, Muro et al. 2006, Xu et al. 2006).

DIAP1 binds to Dronc but does not inhibit it. Instead, DIAP1 promotes Dronc ubiquitination through its C-terminal RING domain (Wilson et al. 2002, Chai et al. 2003). DIAP1 binds Dronc through BIR2 (Meier et al. 2000, Chai et al. 2003). A shallow groove in this domain interacts with a 12-residue sequence in Dronc (114-SRPPFISLNERR-125) located between the CARD domain and the large catalytic subunit (Chai et al. 2003). Interestingly, this sequence is located upstream of documented cleavage sites within Dronc for Drice and Dcp-1 (132-DIVD-135) and for Dronc itself (140-EASE-143), providing several mechanisms by which Dronc activity can become DIAP1 independent. In support of such a model, mutants of Dronc unable to bind DIAP1 show enhanced killing ability when overexpressed (Chai et al. 2003). It will be interesting to determine if Dronc's DIAP1-binding peptide is in fact removed in cells committed to death. Antibodies that recognize only cleaved Dronc (cf. Huh et al. 2004b) would be particularly useful tools for this analysis.

DIAP1 E3 Ligase Activity Has Both Pro- and Antiapoptotic Roles

DIAP1's function as an E3 ubiquitin ligase is essential because point mutations that disrupt this activity result in an embryonic lethal phenotype (Wang et al. 1999, Goyal et al. 2000, Lisi et al. 2000, Yoo et al. 2002). As discussed above, DIAP1 ubiquitinates Dronc and (through whatever mechanism) inactivates it. DIAP1 can also promote the ubiquitination and degradation of other proapoptotic proteins that it binds such as Rpr, discussed below (Olson et al. 2003b). These modifications are antiapoptotic. However, DIAP1 also ubiquitinates itself (Hays et al. 2002,

Holley et al. 2002, Ryoo et al. 2002, Wing et al. 2002b, Yoo et al. 2002). This targets it for proteosome-dependent degradation, and thus DIAP1 has a short half-life of approximately 30 min (Holley et al. 2002, Yoo et al. 2002). In some contexts, this activity is clearly proapoptotic. For example, the half-life of Dronc, an important target, is much longer, approximately 3 h (Yoo et al. 2002). Thus, all other things being equal, anything that disrupts the balance between the synthesis and degradation of DIAP1 and Dronc, such as the inhibition of protein synthesis (which occurs, for example, following infection by many viruses), will result in an increase in the relative levels of Dronc over DIAP1. This increase is sufficient to promote cell death (Yoo et al. 2002). In addition, as discussed further below, the binding of proapoptotic proteins such as Hid to DIAP1 stimulates DIAP1 autoubiquitination and degradation (Yoo et al. 2002), whereas the binding of the mammalian protein Smac/Diablo to XIAP suppresses XIAP autoubiquitination (Creagh et al. 2004, Silke et al. 2004). Thus, the net effect of an IAP's ligase activity depends on the relative levels of *cis* to *trans* ubiquitination, a step clearly under regulatory control.

INITIATOR CASPASE ACTIVATION: UNANSWERED QUESTIONS

Roles for Cytochrome *c*?

An important outstanding question is whether there are upstream regulators of Ark-dependent Dronc activation. In mammals Bcl-2 family proteins regulate the activation of Apaf-1 indirectly by controlling the release of cytochrome *c* from mitochondria. Cytoplasmic cytochrome *c* binds to the WD-40 repeats of monomeric dATP/ATP-bound Apaf-1. This stimulates dATP/ATP hydrolysis to dADP/ADP and a conformational change in Apaf-1 that, in the presence of the physiological levels of dATP/ATP, leads ultimately to the assembly of a large heptameric ring, the apoptosome, consisting of seven dATP and cytochrome-*c*-loaded Apaf-1 molecules (Jiang & Wang 2004). This complex recruits and drives the activation of caspase-9. In contrast, there is little or no evidence to support a similar role for mitochondria and/or cytochrome *c* in Ark- and Dronc-dependent cell death. Ark contains C-terminal WD-40 repeats. Ark was reported early on to bind cytochrome *c* (Kanuka et al. 1999, Rodriguez et al. 1999) and to be shifted into a high-molecular-weight complex in cell extracts in the presence of cytochrome *c* (Dorstyn et al. 2002). However, more recently the structure of a putative *Drosophila* apoptosome has been obtained. This structure consists of eight (rather than the mammalian seven) Ark molecules but in other ways closely resembles the mammalian apoptososome. Yet this complex fails to bind cytochrome *c* (Yu et al. 2006). Furthermore, the addition of cytochrome *c* to *Drosophila* cell extracts results in only modest (an approximately twofold) caspase activation (Kanuka et al. 1999, Dorstyn et al. 2004), even though *Drosophila* cytochrome *c* is able to stimulate caspase activation in extracts from mammalian cells (Dorstyn et al. 2004).

More generally, a recent careful analysis of the requirements for caspase activation in S2 cell extracts found no evidence for the involvement of any mitochondrial components in caspase activation, despite the fact that mitochondrial extracts from these cells could induce robust caspase activation in mammalian cell extracts. Interestingly, in this same system the introduction of a short peptide corresponding to the N-terminal 11 residues of Hid, which disrupts interactions between DIAP1 and Dronc, was sufficient to drive caspase activation (Means et al. 2006). Finally, the downregulation of one or both forms of cytochrome *c* in cells or intact animals using RNAi or mutation has also failed to identify roles for cytochrome *c* in caspase activation (Zimmermann et al. 2002, Dorstyn et al. 2004, Means et al. 2006). Although it is difficult to formally rule out roles for homeopathic quantities of cytochrome *c*, or especially stable

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or sequestered pools of this protein, the above observations show that cytochrome *c*, and mitochondrial components more generally, is unlikely to be a physiological activator of apoptotic caspase activity in flies. In contrast to this large body of work are several reports arguing that cytochrome *c* is required to activate Drice in a nonapoptotic role in *Drosophila* spermatids. The basis for this claim involves the observation that spermatid-specific staining associated with an antibody recognizing cleaved and therefore presumably active versions of Drice (but perhaps other proteins as well) disappears in mutants that lack cytochrome *c* (Arama et al. 2003, 2005). Unfortunately (for this hypothesis), although Drice is clearly important for individualization (Muro et al. 2006), animals that lack any Drice or Dcp-1 protein still show spermatid-specific staining with these antibodies, making unclear the relationship of this staining to caspase activity (Muro et al. 2006). In addition, electron microscopy analysis shows that spermatid development is grossly disrupted in animals that carry mutations in cytochrome *c* (Huh et al. 2004b). Therefore, cytochrome *c* may be required for Drice activation in spermatids. However, this almost certainly reflects an overall developmental requirement for cytochrome *c* to get spermatids to the stage at which caspase activation can occur (not an uninteresting topic), not a molecular mechanism by which caspase activation occurs.

Roles for Bcl-2 Family Proteins?

As discussed above, in *C. elegans* the Bcl-2 family member CED-9 binds CED-4, blocking its ability to activate CED-3 (Yan & Shi 2005). Could Ark activation be regulated similarly in *Drosophila*? *Drosophila* has two multidomain Bcl-2 family members, encoded by *debcl* (also known as *drob1/dborg1/dbok*) and *buffy* (also known as *dborg2*). BH3-domain-only proteins have not been identified. Debcl and Buffy have modest pro- and antiapoptotic activities in various assays (reviewed in Igaki & Miura 2004). Perhaps most striking, the expression of *buffy* has been reported to suppress death owing to the loss of DIAP1 in the embryo (but not in the eye) (Quinn et al. 2003). However, no direct links between Buffy/Debcl and Ark or Dronc, such as physical or strong genetic interactions, have been reported. If Debcl and/or Buffy are important positive/negative regulators of Ark activation, it is particularly curious that mutations in these genes have not been identified in the many genetic screens carried out for the components of the Dronc-DIAP1 cell death pathway. Perhaps they have simply not been hit. However, *Drosophila* Bcl-2 proteins may be more peripheral players that fine-tune specific cell deaths. In any case, it is remarkable (and thus an opportunity) that so little is known about these fly counterparts to the mammalian Bcl-2 family, which has been studied intensively for the past 17 years.

Roles for DIAP1 or Unknown Factors?

Another interesting candidate regulator of Ark-dependent Dronc activation is DIAP1 itself. One simple observation is highly suggestive: Embryos homozygous for the *th6* mutation, which carries a missense mutation that disrupts DIAP1's E3 ligase activity, die. Strikingly, they do not show the massive and ubiquitous activation of the Dronc target caspase Drice (as visualized with a cleaved Drice-specific antibody) seen in DIAP1-null mutants (Wang et al. 1999, Yoo et al. 2002; J.R. Huh & B.A. Hay, unpublished data). Perhaps DIAP1, although no longer able to inhibit active Dronc through its E3 ligase activity, is still able to inhibit Dronc activation. For example, DIAP1 binds to Dronc through sequences located near the Dronc prodomain. This binding may block interactions between Ark and Dronc required for caspase activation, a hypothesis easily tested (but not yet reported) using purified protein in in vitro assays. Alternatively, DIAP1 may normally sequester and/or promote the degradation of proteins that promote Ark-dependent activation of Dronc. Finally, novel proteins may be involved in

regulating Ark-dependent Dronc activation. In either of these latter scenarios, genome-wide RNAi-based screens for suppressors of DIAP1 RNAi–induced cell death in S2 cells may provide a particularly straightforward approach to identifying these molecules.

ACTIVATING CELL DEATH: THE KEY ROLE OF THE RHG FAMILY OF IAP-BINDING PROTEINS

Regardless of whether or how Dronc activation is regulated, for most cells in the fly, cell fate—survival or death—clearly is determined by the relative levels of active caspases and DIAP1 and by the ability of these two classes of proteins to interact with each other. How do cells tip the balance so as to promote cell death? In one essential pathway, caspase-dependent cell death is initiated by the increased expression or release from a sequestering environment of proteins such as Rpr (White et al. 1994), Hid (also known as Wrinkled) (Grether et al. 1995), Grim (Chen et al. 1996), Sickle (Christich et al. 2002, Srinivasula et al. 2002, Wing et al. 2002a), and Jafrac2 (Tenev et al. 2002). These proteins, known collectively as the RHG genes, share only one or perhaps two short regions of homology (Zhou 2005). They promote death, in part, by binding to DIAP1, disrupting its ability to inhibit caspase activity. Wang et al. (1999) first demonstrated this in yeast, in which RHG protein expression induced the death of caspase-expressing cells rescued from death through the expression of DIAP1. More recent work has shown that RHG proteins disrupt DIAP1-caspase interactions through multiple mechanisms (discussed below).

The in vivo roles for most of the RHG genes are just being explored. The deletion of the genomic region that contains *rpr*, *hid*, and *grim* (the H99 deletion) results in the loss of almost all cell death during embryogenesis, providing compelling evidence that, in aggregate, these genes are essential death activators (White et al. 1994). However, single-gene mutants are only available for *hid* (Abbott & Lengyel 1991, Grether et al. 1995), whereas *rpr* alone is eliminated by a pair of overlapping deficiencies (Peterson et al. 2002). Animals that lack either of these genes show some defects in cell death in specific tissues, but many deaths still occur normally. Given these limitations, most in vivo functions attributed to the RHG proteins come from the analysis of phenotypes resulting from overexpression.

RHG Protein Binding to DIAP1 Baculovirus IAP Repeat (BIR) Domains Displaces Other DIAP1-Bound Proteins Such as Caspases

Each of the RHG proteins binds DIAP1 through BIR1 and/or BIR2 (Vucic et al. 1998, Wu et al. 2001, Tenev et al. 2002, Chai et al. 2003, Zachariou et al. 2003, Yan et al. 2004b). This interaction is mediated by a short sequence, the IAP-binding motif (IBM), whose N-terminal residue is alanine (Ala). This motif is present at the N terminus (or neo–N terminus) of each of these proteins (**Figure 2**). For Rpr, Hid, Grim, and Sickle, the N-terminal Ala residue of the IBM is exposed following cleavage of the initial methionine, presumably by methionine aminopeptidase. In the case of Jafrac2, the N-terminal Ala is exposed following the removal of an N-terminal signal sequence as the newly synthesized protein is targeted into the lumen of the endoplasmic reticulum. Rpr and Grim bind equally well to DIAP1 BIR1 or BIR2, whereas Hid, Sickle, and Jafrac2 bind preferentially to BIR2 (Zachariou et al. 2003). In each case the N-terminal RHG tetrapeptide binds a surface groove on the relevant BIR (Wu et al. 2001, Chai et al. 2003, Yan et al. 2004b). More C-terminal residues may also contribute to overall affinity, but the interactions between the tetrapeptide and BIR are essential for function. These same BIR grooves are also used for binding caspases. The neo–N terminus of Drice and Dcp-1 generated following cleavage of the p20 subunit begins with Ala

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and resembles the RHG IBM. N-terminal exposure of this Ala and nearby residues through cleavage is required for these caspases to bind DIAP1 BIR1 (Tenev et al. 2005). Binding of Rpr or Grim to BIR1 competitively displaces the bound caspase. In the case of Dronc, a 12-residue peptide mediates binding to DIAP1 BIR2. This sequence does not resemble the RHG IBM and does not require cleavage for interaction, yet the Dronc-binding pocket on BIR2 coincides with that required for binding to the N-terminal sequences of the RHG proteins. That this peptide binds to the BIR in the reverse orientation explains the above features of the Dronc sequence (Chai et al. 2003). Nonetheless, the consequences of RHG protein binding are the same: Dronc is competitively displaced from its interaction with DIAP1 (Tenev et al. 2002, Chai et al. 2003).

RHG Proteins Stimulate DIAP1 Ubiquitination and Degradation

RHG proteins also regulate DIAP1 levels through ubiquitination. Two mechanisms are likely important: the stimulation of DIAP1 autoubiquitination and the stimulation of DIAP1 ubiquitination by other E3 ligases (Hays et al. 2002, Holley et al. 2002, Ryoo et al. 2002, Wing et al. 2002b, Yoo et al. 2002). Nonubiquitinated proteins found in complex with ubiquitinated proteins targeted to the proteosome typically are not targeted for degradation but instead recycled to the cytoplasm (cf. Johnson et al. 1990, Verma et al. 2001). Therefore, these RHG protein activities are expected to be not only proapoptotic but also catalytic—to the extent that the RHG proteins remain nonubiquitinated when bound to DIAP1. Below we discuss the published observations (some seemingly contradictory) in some detail. We focus on this topic because the regulation of DIAP1 stability appears to be a major mechanism of cell death control. Expression of Hid (in the presence of p35) induces a dramatic decrease in DIAP1 levels in multiple tissues in the fly (Yoo et al. 2002, Huh et al. 2004a). [One group failed to observe Hid-dependent downregulation of DIAP1, but this probably reflects issues with reagent quality (antibodies, Gal4 drivers) (Ryoo et al. 2002).] Hid-dependent downregulation of DIAP1 protein (but not transcript) levels requires that DIAP1 have an intact RING domain (Yoo et al. 2002, Yokokura et al. 2004). Furthermore, versions of DIAP1 (including *th6*) that lack an intact RING domain are better able to protect against Hid-dependent death than is wild-type DIAP1 (Hay et al. 1995, Lisi et al. 2000, Wilson et al. 2002). The addition of full-length Hid to rabbit reticulocyte lysate or embryo extracts stimulates DIAP1 ubiquitination and degradation, and this requires that DIAP1 have an intact RING domain (Yoo et al. 2002). These authors were unable to observe the stimulation of DIAP1 ubiquitination in a purified protein system consisting of purified E1, E2, Ubiquitin, DIAP1, and Hid. Although this may simply reflect difficulties in getting a number of bacterially expressed proteins to behave well together, it may also suggest that other proteins are required.

Together, the above observations are consistent with the hypothesis that Hid stimulates DIAP1 autoubiquitination, but they do not exclude the possibilities that Hid recruits other E3s and that this activity is blocked in the *th6* mutant. In any case, how Hid stimulates DIAP1 ubiquitination (recruitment of an E2 or an E3, induction of a conformational change in DIAP1?) is completely unknown. It is also unknown how MAPK (mitogen-activated protein kinase)-dependent phosphorylation of Hid suppresses its killing activity (Bergmann et al. 1998). Although many mechanisms can be envisioned, it will be interesting to see if the phosphorylation of Hid inhibits its ability to promote DIAP1 degradation.

The expression of Rpr or Grim, along with p35 to inhibit caspase activity, also stimulates DIAP1 degradation. In this case, again using the *th6* point mutant that lacks E3 ligase activity, Yoo and colleagues (2002) found these proteins could stimulate degradation of

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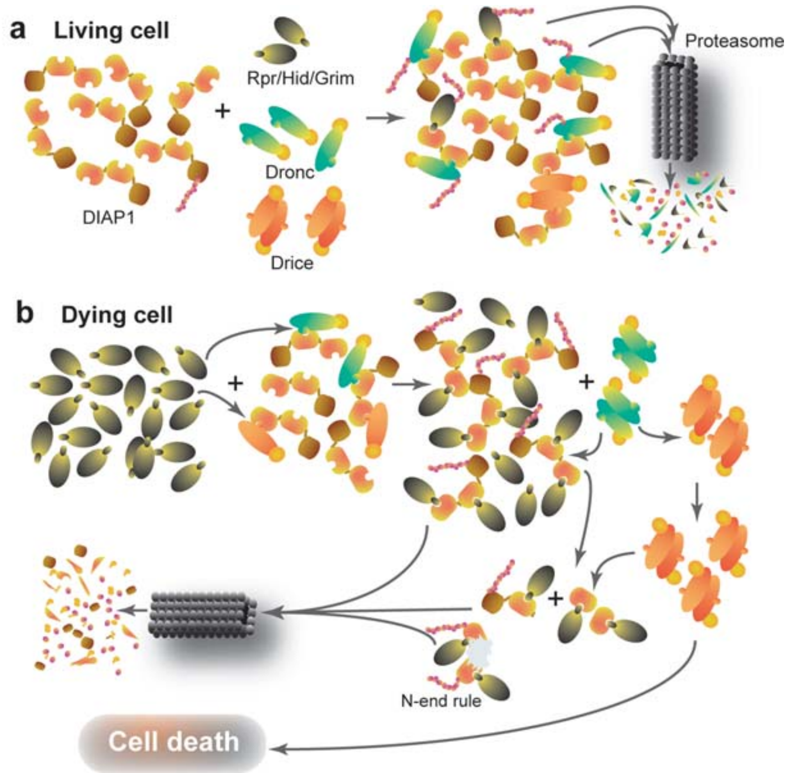

IAP-binding proteins

1) IAP antagonists

Mechanism		Protein	N-terminal sequence	Fly	Mammal
Transcriptional activation		Rpr	MAVAFY...	+	?
Transcriptional activation		Grim	MAIAYF...	+	?
Transcriptional activation		Sickle	MAIPFF...	+	?
Transcriptional activation		Hid	MAVPFY...	+	?
Release from compartment	ER stress →	Jafrac2	AKPED...	+	+
Release from compartment	Mitochondrial stress →	Smac/Diablo	AVPIA...	?	+
Release from compartment	Mitochondrial stress →	Omi-Htra2	AVPSP...	?	+
Cleavage by unknown protease →		GSPT1/eRF3	AKPFV...	+	+

2) Caspases

Protein	N-terminal sequence	Subunits
Caspase-9	ATPFQ...	p10
Caspase-7	AKPDR...	p20
Caspase-7	ANPRN...	p10
Dcp-1	AKGCT...	p20
Drice	ALGSV...	p20
Dronc	SRPPFISLNERR	

DIAP1 in multiple tissues, and in contrast to Hid, this did not require DIAP1 to have a functional RING. This result is particularly striking because in unstimulated cells the *th6* protein has a dramatically increased stability as compared with wild-type DIAP1 (see also Yokokura et al. 2004). In contrast to these observations, Ryoo and colleagues (2002) failed to observe Rpr-dependent stimulation of DIAP1 degradation in embryos transheterozygous for two DIAP1 mutants. Although an observation of Rpr-dependent degradation of *th6* DIAP1, which cannot ubiquitinate itself, trumps a failure to observe degradation of other DIAP1 mutant proteins, these differences may provide information about the regulation of DIAP1. Ryoo and colleagues (2002) did not include p35 in their experiments (e.g., their figure 3f). Therefore, unleashed caspase activity may have cleaved DIAP1 and thereby stabilized fragments of DIAP1 recognized by their antibodies. Alternatively, and/or in addition, caspases activated by *rpr* may have inactivated other substrates required for DIAP1 degradation. For example, Adrain et al. (2004) showed that caspase activity inhibits the *Drosophila* proteasome.

It may also be important that Ryoo and colleagues (2002) used embryos transheterozygous for two different mutations: *diap1*$^{33-1S}$, a RING finger deletion, and *diap1*$^{22-8S}$, a RING domain point mutant distinct from *th6*. RING domains mediate not only ubiquitination but also protein-protein interactions. Perhaps most relevant for this discussion, interactions between mammalian cIAP1 and XIAP are mediated by their RING domains, and these interactions are required for cIAP1 to promote XIAP degradation (Silke et al. 2005). Interactions between DIAP1 and other E3 ligases and/or RING domain proteins have not been identified (it is not clear if they have been looked for). Nonetheless, one or both of the mutant proteins utilized by Ryoo and colleagues (2002) may lack the ability to recruit other proteins necessary for DIAP1 ubiquitination in *trans*, and this may explain the failure of these DIAP1 proteins to undergo Rpr-dependent ubiquitination.

Rpr can stimulate DIAP1 ubiquitination in some heterologous cell extracts (reticulocyte lysates or Xenopus egg extracts) (Holley et al. 2002, Ryoo et al. 2002). However, because extracts contain many cellular proteins, these observations really do not tell us if the stimulation of DIAP1 ubiquitination observed is a result of autoubiquitination or ubiquitination in *trans*. The possibility of ubiquitination

Figure 2

DIAP1 and the regulation of cell death. (*a*) In unstressed cells, low levels of RHG proteins, Dronc, and activated Drice bind through short IAP-binding motif (IBM) peptides to surface grooves on one or both DIAP1 baculovirus IAP repeat (BIR) domains. Proapoptotic proteins bound by DIAP1 are inhibited and/or ubiquitinated and in at least some cases are targeted for degradation by the proteosome. These buffering functions of DIAP1 are required for cell survival. When unbound, DIAP1 is autoubiquitinated and targeted for degradation. This short half-life (a potentially proapoptotic feature of DIAP1) may be the price paid for having robust ubiquitination activity against continuously generated toxins such as active Dronc and Drice. A short half-life should also facilitate rapid changes in DIAP1 protein levels in response to changes in translation or transcription. (*b*) In cells targeted for death, RHG protein levels increase. These proteins bind to the same sites on DIAP1 BIR domains as caspases, thereby displacing these proteins and liberating their activity. The binding of RHG proteins to DIAP1 also directly stimulates DIAP1 autoubiquitination or ubiquitination by other ligases. Dronc can cleave DIAP1 between BIR1 and BIR2. Cleavage of full-length DIAP1 or the Dronc-cleaved DIAP1 BIR1 fragment by Drice creates a new DIAP1 N terminus that may target DIAP1 for degradation. (*Bottom*) A number of proteins have been found to bind to IAP BIR domains through a short IBM motif exposed by the removal of more N-terminal sequences (reviewed by Yan & Shi 2005). Proteins listed as IAP antagonists have been proposed or shown to inhibit IAP-anticaspase function; caspases also bind to IAPs utilizing IBMs exposed following cleavage. In the case of Dronc, the peptide binds in the reverse orientation as compared with the other proteins, and cleavage is not required. ER, endoplasmic reticulum.

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in *trans* is strengthened by several recent observations with Grim, which in all other assays behaves identically to Rpr. When expressed in mammalian cells, Grim can stimulate the ubiquitination and degradation of XIAP. This requires interaction between the two proteins but does not require XIAP's ligase activity, thereby demanding the recruitment of a second E3 (Silke et al. 2004). In addition, although Grim can bind the E2 UbcD1 and stimulate DIAP1 ubiquitination in *Drosophila* embryo extracts supplemented with UbcD1, Grim fails to stimulate DIAP1 ubiquitination in an in vitro assay containing only purified proteins: DIAP1, UbcD1, E1, and Ubiquitin (Yoo 2005). Together with the positive in vivo observations of *th6* DIAP1 degradation by Rpr and Grim reported by Yoo and colleagues (2002), these observations seem to require that Rpr and Grim stimulate DIAP1 degradation (at least in vivo) in *trans*. Many components of the ubiquitin pathway have been identified as regulators of RHG-mediated cell death. Examples include the ubiquitin activating enzyme (*uba1*), two components of an SCF-type E3 ubiquitin ligase (*skpA* and a novel F-box gene, *morgue*), and the deubiquitinating enzyme *fat facets* (Hays et al. 2002, Ryoo et al. 2002, Wing et al. 2002b). It is not clear, mechanistically, how any of these molecules function to regulate RHG-dependent degradation of DIAP1. In any case, the screens that identified these genetic modifiers were all quite small, suggesting that there is a lot to learn about how DIAP1 stability is regulated.

Other RHG Protein Proapoptotic Activities

The above discussion shows that the IBM motif is essential for RHG proteins to disrupt IAP-caspase interactions, and this activity is often sufficient to induce death. However, some RHG proteins may also have other proapoptotic activities. Rpr and Grim can also inhibit global protein translation (Holley et al. 2002, Yoo et al. 2002, Tait et al. 2004, Colon-Ramos et al. 2006). In addition, the overexpression of versions of Rpr or Grim that lack the N-terminal IBM can kill *Drosophila* and mammalian cells (Chen et al. 1996, 2004; Vucic et al. 1997; Claveria et al. 1998, 2002, 2004; McCarthy & Dixit 1998; Wing et al. 1998, 2001; Thress et al. 1999; Tait et al. 2004). Sequences that are necessary and/or sufficient for killing in these contexts map to an internal region of sequence conservation shared between Rpr, Grim, and Sickle throughout the dipteran lineage (Zhou 2005). This region (or regions), defined by several groups through different assays, and with different boundaries, is known variously as the Trp-block (Wing et al. 2001), the GH3 domain (Claveria et al. 2002), or the R3 domain (Chen et al. 2004). Sequences in this same region are also important for Rpr's ability to promote the degradation of DIAP1 (Olson et al. 2003a), but the relationship between these activities is unknown. The expression of Grim in vertebrate cells, or the introduction of Rpr into extracts of these cells, can promote caspase-dependent cell death mediated by Trp/GH3/R3, not IBM sequences, although in at least one other mammalian cell type expression of Grim does not induce cell death (Silke et al. 2004). These deaths are caspase dependent and involve Trp/GH3/R3 domain–dependent targeting of RHG proteins to mitochondria and the stimulation of cytochrome *c* release. In contrast, a number of observations in *Drosophila* suggest that IBM-independent effects of RHG protein expression on cell death are at least in part caspase independent in that they are not inhibited by the expression of DIAP1, p35, or a dominant-negative version of Dronc (Wing et al. 1998, 2001; Chen et al. 2004). Rpr and Grim's ability to inhibit global translation may contribute to some of these effects, but translational inhibition has not been observed in all contexts in which IBM-independent death was induced (Chen et al. 2004), suggesting roles for other activities (which are currently unknown).

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What is the significance of IBM-independent killing activity, particularly the caspase-independent activities identified in flies? The problem is that the expression of p35 or DIAP1 efficiently blocks normally occurring cell death (which is RHG dependent) as well as death induced by the expression of full-length RHG proteins. Thus, although death-inducing activities can be demonstrated following massive overexpression of RHG proteins that lack IBM sequences, the significance of these effects is questionable. Observations demonstrating a decreased killing ability of IBM-containing RHG proteins with mutated Trp/GH3/R3 sequences would seem to provide compelling evidence for such activities (cf. Claveria et al. 2002). However, because sequences in this region are also required for IBM-dependent degradation of DIAP1, it is unclear whether the effects observed should be ascribed to an independent activity or just decreased ability to downregulate DIAP1. One possible resolution of this paradox proposes that IBM-independent activities only become exposed following caspase-dependent cleavage of RHG proteins, which would be blocked in the presence of p35 or DIAP1 (Chen et al. 2004). Some evidence consistent with cleavage has been obtained (Chen et al. 2004). A key test of this hypothesis will involve measurements of the killing activity of uncleavable versions of RHG proteins that can still promote IBM-dependent DIAP1 degradation.

How Many RHG-Like IAP-Binding Proteins Are There?

The functions of the RHG proteins are regulated in a number of ways. The most prominent of these is indicated in **Figure 3**. The examination of a modest number of confirmed caspase substrates suggests that caspase cleavage exposes IBM-like motifs in many proteins (Hell et al. 2003). If true in vivo, this may serve as a form of positive feedback that sensitizes cells to further caspase activation. Alternatively, or in addition, the exposure of an IBM motif on these proteins may serve primarily to target aberrant fragments for ubiquitination and degradation when caspase activity is activated at low levels (perhaps for other purposes) in otherwise healthy cells. Most mammalian and *Drosophila* proteins with IBM motifs were identified as IAP-binding proteins secondary to their identification as cell death regulators. Genome-scale two-hybrid screens have been carried out in *Drosophila*, but they have failed to identify any of the known IAP-binding proteins (cf. Giot et al. 2003, Formstecher et al. 2005). Unfortunately this failure is expected given that most two-hybrid screens utilize prey fusion proteins in which the N terminus of the cellular protein is masked by the transcriptional activation domain appended to it. Two IAP-binding proteins, GSPT1/eRF3 in mammals and Jafrac2 in flies, were identified through direct immunoprecipitation experiments from cells (Tenev et al. 2002, Hedge et al. 2003). But these experiments involved the use of healthy, unstimulated cells. Notably absent from the literature are reports of systematic immunoprecipitation screens, in cells or tissues exposed to "interesting" cell death stimuli, for proteins that can bind IAPs. In short, there is no reason to believe that the full complement of interesting IAP-binding proteins has been identified.

MULTIPLE REGULATORS OF CASPASE-DEPENDENT CELL DEATH ARE IMPORTANT IN NONAPOPTOTIC CONTEXTS

Many nonapoptotic roles for caspases have been identified in mammals. These include innate immunity, cell differentiation, proliferation, and survival (as opposed to death) (Launay et al. 2005). Nonapoptotic roles for caspases and caspase regulators are also beginning to be identified in *Drosophila* (**Figure 4**). Importantly, each cell type involved can be induced to undergo caspase-dependent cell death. Therefore, mechanisms to channel or

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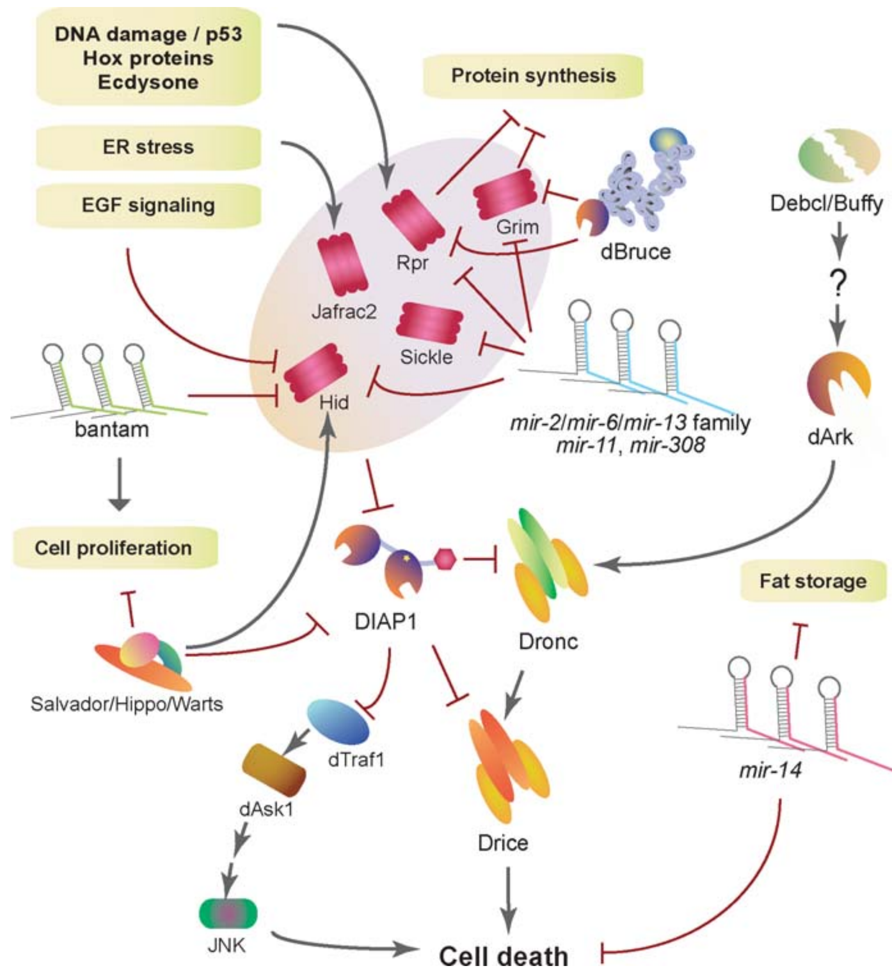

Figure 3

Regulators of caspase-dependent cell death in *Drosophila*. Members of the RHG family of DIAP1-binding proteins are regulated through multiple pathways. MicroRNAs are important regulators of cell death in the fly. *Mir-11*, *mir-308*, and members of the *mir-2/mir-6/mir-13* family of microRNAs are likely to regulate the translation of Rpr, Hid, Grim, and Sickle in some contexts (Stark et al. 2003, Brennecke et al. 2005, Grun et al. 2005, Leaman et al. 2005). Hid translation is also negatively regulated by the bantam microRNA, which also promotes cell proliferation through unknown mechanisms (Brennecke et al. 2003). The *mir-14* microRNA inhibits cell death and fat storage through unknown mechanisms (Xu et al. 2003, 2004). Hid is negatively regulated by the epidermal growth factor (EGF) receptor/Ras/MAPKinase pathway through phosphorylation (EGF signaling) and positively regulated by the tumor suppressors Hippo, Salvador, and Warts (Edgar 2006). Rpr- and Grim-dependent cell killing is inhibited by the large, BIR- and UBC-containing protein Bruce (Vernooy et al. 2002). All the RHG family members bind to DIAP1 and inhibit its antiapoptotic activities. In a second pathway Rpr binding to DIAP1 results in the stabilization of the *Drosophila* tumor necrosis factor–associated factor 1 (Traf1), which promotes the activation of the *Drosophila* apoptosis signal-regulating kinase 1 (Ask1). Ask1 activation leads, through unknown mechanisms (the *two arrows*), to c-Jun N-terminal kinase (JNK) activation (Kuranaga et al. 2002). JNK activation promotes cell death in some but by no means all contexts. In addition, at least Rpr and Grim also have DIAP1-independent proapoptotic activities, one of which is the general inhibition of translation through the inhibition of start codon recognition during translation initiation (Colon-Ramos et al. 2006). ER, endoplasmic reticulum.

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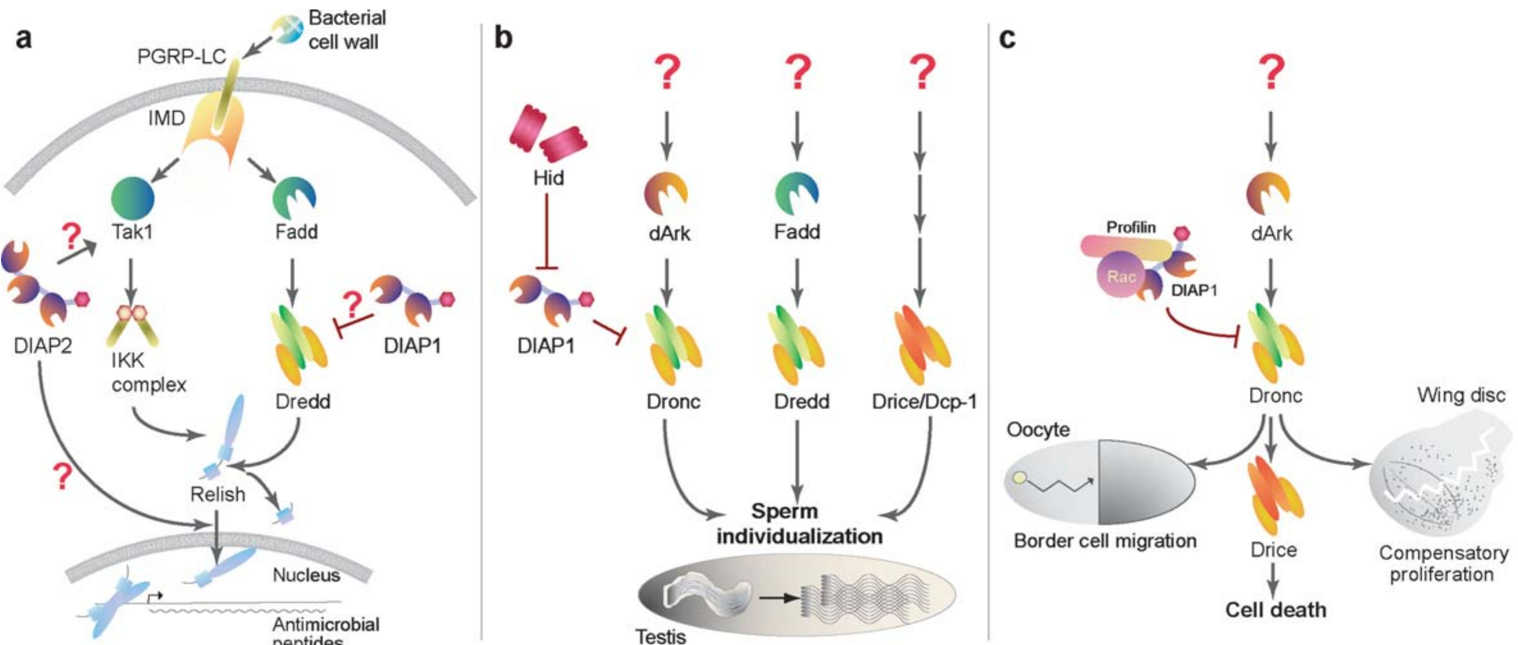

Figure 4

Nonapoptotic roles of key cell death regulators in *Drosophila*. (*a*) The innate immune response to gram-negative bacterial infection is initiated when bacterial cell-wall components are recognized by receptors such as peptidoglycan recognition protein-LC (PGRP-LC). The activation of the receptor initiates signaling cascades transduced by IMD through two independent downstream branches. In one pathway the kinase Tak1 activates the IKK complex, which phosphorylates the NF-κB homolog Relish. In a second pathway the adaptor Fadd binds the caspase Dredd, promoting its activation. Dredd cleaves Relish, thereby releasing an inhibitory domain and promoting its nuclear translocation. Nuclear Relish then promotes the transcription of antimicrobial peptide genes. Both pathways are important for Relish activation (Hultmark 2003). DIAP2 was identified as a potent inhibitor of Rpr-, Hid-, and Grim-dependent cell death (Hay et al. 1995). Two recent reports show that DIAP2 is required for the innate immune response to gram-negative bacteria in S2 cells, although they argue for different sites of action (*question marks*) (Gesellchen et al. 2005, Kleino et al. 2005). Our own observations, using animals deleted for *diap2*, indicate that DIAP2 is required for most, but not all, Relish cleavage (J.R. Huh & B.A. Hay, unpublished data). DIAP1 is an inhibitor of all tested *Drosophila* caspases. It will be interesting to know if Dredd can be inhibited by DIAP1, and if so, how Dredd avoids inhibition during an immune response. (*b*) During the late stages of spermatogenesis, spermatids, which develop within a syncytium, must become separated from each other. This individualization involves the activity of multiple caspase activators, including Hid, Ark, and Fadd, and the caspases Dronc, Dcp-1, Drice, and Dredd (Huh et al. 2004b, Arama et al. 2005, Muro et al. 2006). The mechanisms by which these caspase cascades are activated, and their targets cleaved, are unknown. (*c*) During the migration of somatic follicle cells known as border cells, Profilin and Rac interact with DIAP1, which regulates Dronc activity. Dronc participates in, but is not absolutely required for, border cell migration (Geisbrecht et al. 2004). When cells in the fly wing disc die as a result of stress (induced by heat or X-ray irradiation), they are replaced by neighboring cells, which undergo compensatory proliferation. This process helps to maintain a constant tissue size. Dronc activity is required for compensatory proliferation, although where and how Dronc acts is unknown (Huh et al. 2004a). Most recently caspase activity has been implicated in neural precursor development (not shown) (Kanuka et al. 2005).

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regulate caspase activity must exist so as not to induce cell death. In each case the following specific questions arise: (*a*) What are the pathways that mediate caspase activation? Are they the same as those used during apoptosis signaling or distinct? (*b*) How are substrates chosen? Are they the same as those cleaved in apoptotic contexts but cleaved at lower levels? Or are they distinct? (*c*) If the substrates are distinct, what is the basis for caspase targeting to one set of substrates or the other? (*d*) Once an apoptotic caspase becomes activated in a nonapoptotic role, how is its activity terminated? Answers to these questions may have interesting implications for understanding the basis for, and treatment of, diseases such as cancer. The inhibition of cell death is an obligatory step for tumor formation, and all tumor cells can likely be induced to undergo caspase-dependent cell death (Green & Evan 2002). IAPs provide one mechanism for inhibiting apoptotic caspase activity. But if active caspases could be sequestered away from their apoptotic targets, or inhibited through other mechanisms, the same goal might be achieved—the prolongation of cell survival.

The differentiation of mammalian megakaryocytes and *Drosophila* spermatids may provide examples of sequestration at work. Thus, in maturing megakaryocytes, active caspase-3 is found in a punctate cytoplasmic distribution, but when these cells become apoptotic, active caspase-3 becomes diffuse, suggesting a model in which the release from sequestration provides access to new substrates (De Botton et al. 2002). Similarly, during *Drosophila* spermatid differentiation, Hid and active Dronc (as visualized with a cleaved-Dronc-specific antibody) are localized to punctate structures at sites of individualization, and genetic evidence indicates they are both important for this process (Huh et al. 2004b). The Dronc target caspase Drice is also present in spermatids and is important for their differentiation (Muro et al. 2006), but genetic evidence suggests that Drice is not activated, at least primarily, by Dronc (Huh et al. 2004b). Importantly, Drice can kill spermatids when expressed in the testis in a constitutively active form, indicating that apoptotic substrates are present in these cells (J.R. Huh & B.A. Hay, unpublished data). Interestingly, however, spermatid-specific expression of a hyperactive version of the Drice-activating caspase Dronc, Dronc^{F118}, that cannot bind or be inhibited by DIAP1 and that kills retinal cells much more efficiently than does wild-type Dronc (cf. Chai et al. 2003), fails to kill spermatids (J.R. Huh & B.A. Hay, unpublished data). All together, these observations suggest that Dronc activation and/or activity in spermatids are highly constrained so as to prevent induction of apoptosis. Genetic screens for mutants in which excess caspase activation and/or cell death occur during nonapoptotic processes that utilize apoptosis-inducing caspases provide one approach to identifying these molecules. Screens for proteins that bind active versions of caspases provide a second, underreported, approach.

PROSPECTS

A basic picture of one important caspase-dependent cell death pathway, involving interactions between RHG proteins, Ark, Dronc, Drice, and DIAP1, has emerged. Many questions remain about how this pathway is activated and how the activities of its components are regulated. All the interactions identified between members of this pathway also occur between mammalian counterparts of these proteins. However, the relative importance of specific points of regulation (the activation of Apaf-1/Ark, the inhibition of IAP function, the role of the Bcl-2 family proteins) differs between the systems. A focus in *Drosophila* on these (apparent) points of divergence is likely to provide unique mechanistic insights into what are in fact conserved mechanisms of death regulation. Many cell deaths can still occur when the canonical Dronc/DIAP1 pathway is disrupted, indicating that other pathways, caspase dependent and independent,

activated in parallel or independently, are also important. Finally, *Drosophila* provides several excellent examples of nonapoptotic roles for apoptotic caspases. Understanding how cells survive the activation of these caspases should identify novel mechanisms of caspase regulation. Central to all this future work are the many genetic approaches available in the fly (Adams & Sekelsky 2002, St. Johnston 2002). This is because genetic approaches are fundamentally function based and they make few assumptions about the kinds of molecules and mechanisms that regulate cell death. Many different kinds of screens for cell death regulators can be carried out, but few of these roads have been well traveled or traveled at all (Hay et al. 2004). There will undoubtedly be many interesting sites to see along the way.

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ACKNOWLEDGMENTS

This work was supported by NIH grant GM57422 to B.A.H. and NIH grants NS048396 and NS042580 to M.G. M.G. is a Sloan Foundation Fellow. The authors thank J.R. Huh for help with the figures and J.R. Huh, C-H. Chen, and I. Muro for providing unpublished observations and comments on the manuscript.

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Annual Review of Cell and Developmental Biology

Volume 22, 2006

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