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Unrestrained caspase-dependent cell death caused by loss of *Diap1* function requires the *Drosophila* Apaf-1 homolog, *Dark*

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In mammals and *Drosophila*, apoptotic caspases are under positive control via the CED-4/Apaf-1/Dark adaptors and negative control via IAPs (inhibitor of apoptosis proteins). However, the *in vivo* genetic relationship between these opposing regulators is not known. In this study, we demonstrate that a *dark* mutation reverses catastrophic defects seen in *Diap1* mutants and rescues cells specified for *Diap1*-regulated cell death in development and in response to genotoxic stress. We also find that *dark* function is required for hyperactivation of caspases which occurs in the absence of *Diap1*. Since the action of *dark* is epistatic to that of *Diap1*, these findings demonstrate that caspase-dependent cell death requires concurrent positive input through Apaf-1-like proteins together with disruption of IAP–caspase complexes.

Keywords: Apaf-1/apoptosis/*Drosophila*/IAP/PCD

Introduction

Apoptosis, or programmed cell death (PCD), is a cell deletion mechanism that is critical to metazoan survival. During development, PCD is essential for adjusting the number of cells within or between populations of cells, for the elimination of non-functional cells and for the sculpting of organs and tissues (reviewed in Jacobson *et al*., 1997; Vaux and Korsmeyer, 1999; Meier *et al*., 2000a). Physiologically, PCD is also pivotal in the maintenance of cell homeostasis and for host defense against viral pathogens. In addition, the misregulation of apoptosis is implicated in human diseases such as cancer and neurodegenerative-associated disorders (reviewed in Thompson 1995).

Studies initiated in the nematode, *Caenorhabditis elegans*, established that PCD is under genetic control and led to the identification of three essential components of the apoptotic pathway: *ced-3*, *ced-4* and *ced-9* (reviewed in Metzstein *et al*., 1998). Homologs of these 'core' apoptotic components are conserved throughout evolution along with many other activators, effectors and inhibitors of cell death. CED-3 represents the founding member of the proapoptotic cysteine proteases known as caspases (Yuan *et al*., 1993), whereas the antiapoptotic component, CED-9, shows structural and functional homology to the *Drosophila* and vertebrate Bcl-2 family of proteins (reviewed in Gross *et al*., 1999; Chen and Abrams, 2000). The third gene product, CED-4, bears homology to vertebrate Apaf-1 (reviewed in Hengartner, 1997) and *Drosophila dark*/*Hac-1*/*Dapaf-1* (Kanuka *et al*., 1999; Rodriguez *et al*., 1999; Zhou *et al*., 1999) and is important in promoting apical caspase activation via the 'apoptosome' (Zou *et al*., 1999; Hengartner, 2000). The caspases, in turn, mediate PCD by cleaving selected intracellular proteins, including proteins of the nucleus, cytosol and cytoskeleton, thereby disabling important cellular processes and breaking down structural components of the cell (Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997).

Molecular genetic studies of PCD in *Drosophila* led to the identification of three additional important cell death initiators known as *reaper*, *grim* and *hid*. The genes encoding these PCD initiators are linked within an ~300 kb genomic interval referred to as the Reaper interval (reviewed in McCall and Steller, 1997; Rodriguez *et al*., 1998; Abrams, 1999). During embryonic development, most, if not all, cells are specified for PCD solely or combinatorially by *reaper*, *grim* and/or *hid* (White *et al*., 1994; Grether *et al*., 1995; Chen *et al*., 1996). These cell death initiators contain a short region of homology at the N-terminus, referred to as the RHG motif (Zhou *et al*., 1997), which is sufficient for binding to at least two of the *Drosophila* inhibitors of apoptosis proteins (IAPs): Diap1/Thread and Diap2 (Vucic *et al*., 1997, 1998; Kaiser *et al*., 1998; Lisi *et al*., 2000). In addition, the RHG of Reaper, Grim and Hid is capable of inducing apoptosis on its own (Vucic *et al*., 1998), so it has been proposed that disruption of caspase–IAP interactions through this domain may be of central importance in order for *reaper*, *grim* and *hid* to induce PCD (Meier and Evan, 1998; Goyal *et al*., 2000; Hay, 2000; Wu *et al*., 2001). In agreement with this model, Diap1 binds directly to at least two (i.e. drICE and Dronc) of the seven fly caspases and inhibits their cell killing activity (Wang *et al*., 1999; Meier *et al*., 2000b). Furthermore, caspase-induced killing in yeast can be blocked by co-expression of Diap1, and this effect can be reversed by co-expressing either Reaper, Grim or Hid (Wang *et al*., 1999). These interactions support a 'liberation' model in which the cell death activators control PCD by releasing caspases from IAP-mediated repression (Miller, 1999; Wang *et al*., 1999; Meier *et al*., 2000b). Consistent with this hypothesis, most, if not all, cells in *Diap1* null animals die with signs of apoptotic DNA fragmentation early during embryogenesis (Wang *et al*., 1999; Goyal *et al*., 2000; Lisi *et al*., 2000). Because IAPs bind activated rather than the zymogenic forms of caspases, absence of these inhibitors in *Diap1* null animals could release pre-existing enzymes from an otherwise repressed condition. Since zymogens exhibit intrinsic auto-activation properties and,

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under the right conditions, can induce cell death on their own (Salvesen and Dixit, 1997), caspase auto-activation followed by cascades of proteolysis could account for apoptosis in the absence of IAPs.

While it is clear that the balance of opposing regulatory forces determines the status of caspase activity in a given cell, the relative physiological contribution from positive regulators (Apaf-1/CED-4/Dark) and negative regulators (the IAP family) remains ill defined. In the worm, the mouse and the fly, mutations in positive regulators cause clear cell death-defective phenotypes, but this is not consistently true with respect to the negative regulators. Though absence of a single IAP gene promotes catastrophic dysregulation of caspases and apoptotic phenotypes in *Drosophila* (Wang *et al*., 1999; Lisi *et al*., 2000), surprisingly, neither of the two IAP homologs in *C.elegans* appear to function in cell death (Fraser *et al*., 1999). Murine IAPs can exert profound inhibitory effects *in vitro* and when overexpressed in cultured cells (Deveraux and Reed, 1999), but genetic knockouts of murine IAPs have only mild (or no) phenotypes, perhaps reflecting redundant functions among mammalian IAP family members (Holcik *et al*., 2000; Harlin *et al*., 2001). The *Drosophila* model is thus uniquely suited to assess the relationship between these positive and negative regulators of caspase action.

Loss of *dark* function is associated with cell death-defective phenotypes opposite to *Diap1*, and genetic evidence indicates that *dark* is also important for *reaper*, *grim* and *hid* death pathways. However, the *in vivo* genetic relationship between *dark*-dependent caspase activation and *Diap1*-dependent caspase inhibition is not known and, consequently, the possible connection between *dark*-associated functions and *reaper*, *grim* and *hid* remains unclear. In this study, we examine the apoptotic role of *dark* and determine its epistatic relationship to *Diap1*. Our results show that *dark* functions downstream or parallel to *Diap1*, arguing against the 'liberation' models, which postulate that IAPs alone are a pivotal determinant of whether a cell will die or live in *Drosophila* (Goyal *et al*., 2000; Meier *et al*., 2000b; Song *et al*., 2000). Instead, we show that important cell death-inducing cues must also converge upon *dark* in order for PCD to occur. These observations support a model of PCD whereby concurrent positive input, in the form of Dark-dependent caspase activity, occurs together with the disruption of IAP–caspase complexes to specify the apoptotic fate.

Results

Programmed cell death specified by reaper, grim and hid requires dark function

We examined the pattern of PCD within the embryonic central nervous system (CNS) midline glia of *dark* mutants (see Materials and methods for comments on *dark* alleles) using the marker strain P[1.0slit-*lacZ*] (Zhou *et al*., 1995). During stages 16–17 of embryogenesis, this marker labels three midline glia per segment in the CNS of wild-type embryos (Zhou *et al*., 1995; Dong and Jacobs, 1997; Figure 1A). However, there is a significant increase in the number of midline glia (~8 cells/segment) present in *dark*^{CD4} mutants (Figure 1B). Thus, at least five supernumerary *dark* cells per segment (and sometimes many

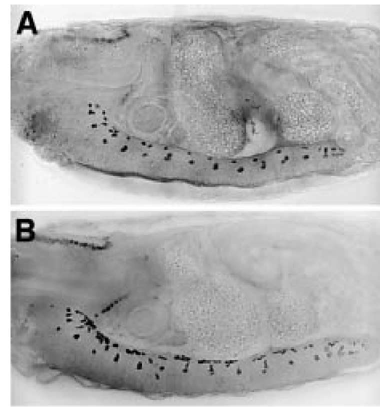

Fig. 1. Cells specified for apoptosis by genes in the *reaper* interval survive in the absence of *dark* function. Shown are anti-β-galactosidase staining of a stage 17 wild-type (**A**) and *dark*^{CD4} (**B**) mutant embryo bearing the P[1.0slit-*lacZ*] marker chromosome. Control *yw* embryos have approximately three P[1.0slit-*lacZ*]-expressing cells per segment at this point in development, whereas similarly staged *dark*^{CD4} mutants have ~8 cells/segment.

more) are not subject to a TUNEL-independent form of cell death nor are they delayed in their death program as reported for some cells in the *Apaf-1* knockout mouse (Cecconi *et al*., 1998; Yoshida *et al*., 1998). Since 9–12 embryonic midline glia survive in Reaper interval [i.e. Df(3L)H99] mutants (Zhou *et al*., 1995; Dong and Jacobs, 1997), our data indicate that Dark is essential for most, if not all, cell death occurring in lineages labeled by this marker.

dark is required for radiation-induced apoptosis mediated by reaper, grim and hid

We next asked whether *dark* might also play an essential role in damage-induced apoptosis mediated by *reaper*, *grim* and/or *hid* (White *et al*., 1994). We therefore examined the incidence of TUNEL-labeled cell death in wild-type (*yw*) and *dark* mutant embryos exposed to ionizing radiation, using previously described irradiation protocols that also induce the *reaper* gene (Nordstrom *et al*., 1996; Nordstrom and Abrams, 2000). As shown in Figure 2A, stage 9 wild-type embryos show widespread, damage-induced cell death throughout the embryo, whereas *dark* mutant embryos treated in parallel show very few and sparsely distributed apoptotic cells (Figure 2B and C). Thus, in addition to physiological PCD, *dark* plays a central role during pathological apoptosis engaged by *reaper*, *grim* and/or *hid*. Furthermore, consistent with the previously reported induction of a *dark*-enhancer trap reporter by UV irradiation (Zhou *et al*., 1999), we observed that expression of *dark* RNA itself is acutely responsive to ionizing radiation (Figure 2D).

dark is required for Diap-1-enhanced cell killing in the eye

Directed expression of *reaper*, *grim* and *hid* under the retina-specific GMR promoter induces widespread apoptotic death in the eye disc, producing a small/rough eye phenotype in adult flies (Grether *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996). Mutations at *dark* and *Diap1* modify these cell death phenotypes in opposite ways; loss

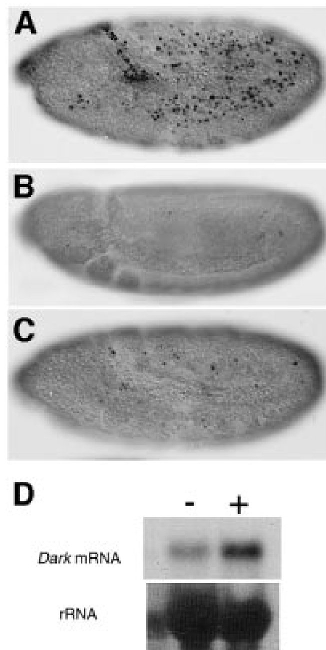

Fig. 2. *dark* is required for damage-induced apoptosis mediated by *reaper*, *grim* and/or *hid*. Nomarski micrographs of stage 9 wild-type (A) or *dark* (B and C) embryos labeled with TUNEL after X-ray treatment. (**A**) A wild-type embryo showing prominent cell death throughout. (**B** and **C**) In contrast, comparably staged, X-ray-treated *dark* embryos show little cell death after radiation treatment. Note that embryos bearing the Df(3L)H99 deletion (i.e. mutant for *reaper*, *grim* and *hid*) are similarly insensitive to X-ray-induced apoptosis (White *et al*., 1994; data not shown). (**D**) The top panel shows a northern blot of control (–) and X-ray-treated (+) wild-type embryos using a *dark* probe. Relative to control embryos, there is a dramatic increase of *dark* RNA in the X-ray-treated embryos. The lower panel depicts the same blot stained with methylene blue showing that similar amounts of total RNA were loaded in each lane.

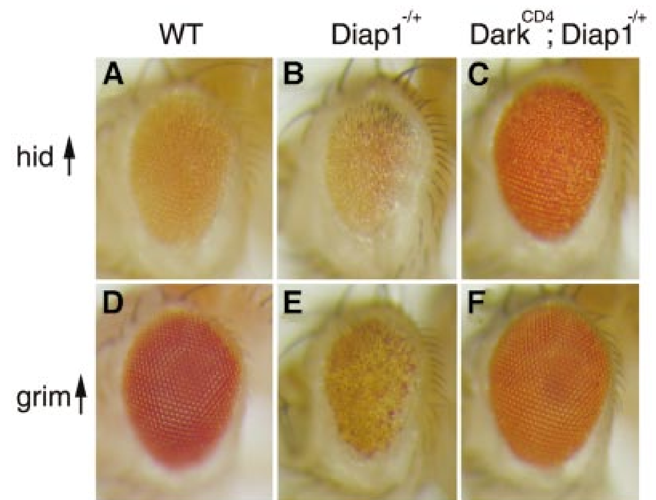

Fig. 3. *Diap1*-mediated enhancement of *hid*- and *grim*-induced cell killing is reversed by loss of *dark* function. Light microscopy micrographs of (**A**) GMR-*hid*-1M/+, (**B**) GMR-*hid*-1M/+; $Diap1^5$/+, (**C**) $dark^{CD4}$, GMR-*hid*-1M/$dark^{CD4}$; $Diap1^5$/+, (**D**) GMR-*grim*-1/+, (**E**) GMR-*grim*-1/+; $Diap1^5$/+ and (**F**) $dark^{CD4}$, GMR-*grim*-1/$dark^{CD4}$; $Diap1^5$/+ fly eyes. (B) A fly wild-type for *dark* and heterozygous for the *Diap1* loss-of-function allele, $Diap1^5$, shows enhancement of GMR-*hid*-1M cell killing. (C) In a fly homozygous for $dark^{CD4}$, the $Diap1^5$ mutation fails to dominantly enhance *hid*-induced cell killing. (E) $Diap1^5$ enhances GMR-*grim*-1 retinal apoptosis in a fly wild-type for *dark*. (F) In contrast, $Diap1^5$ fails to enhance *grim*-induced cell killing in a $dark^{CD4}$ mutant fly.

Table I. A *dark* mutation rescues defective egg laying caused by lesions at *DIAP1*

Female genotype[a]	Fail to lay eggs	*n*[b]
th^6/th^8	91%	34
$dark^{CD4}$/+; th^6/th^8	69%	54
$dark^{CD4}/dark^{CD4}$; th^6/th^8	47%	63

[a]Single pair matings with *yw* males were set up using females of the genotype shown.
[b]Individual vials with females were monitored for up to 14 days for eggs in food.

of *dark* gene function suppresses cell killing by GMR-*hid* and GMR-*grim* transgenes (Rodriguez *et al*., 1999), and hypomorphic *Diap1* alleles act as strong dominant enhancers of these same transgenes (Hay *et al*., 1995; Goyal *et al*., 2000; Lisi *et al*., 2000). To examine further the influence of *dark* and *Diap1* upon apoptotic signaling and establish the order of gene action in this context, we undertook a series of epistasis studies. We tested whether *dark* alleles modify *Diap1*-mediated enhancement of Hid- or Grim-induced cell killing by introducing GMR-*hid* or GMR-*grim* tester strains into a genetic background heterozygous for *Diap1* and homozygous for the $dark^{CD4}$ mutation. While the $thread^5$ ($Diap1^5$) loss-of-function mutation is still an effective dominant enhancer of GMR-*grim*- or GMR-*hid*-induced cell killing in flies heterozygous for *dark* (data not shown), $Diap1^5$ fails to modify cell killing of these same transgenes in flies homozygous for *dark* (Figure 3, for *hid* killing compare B and C, for *grim* killing compare E and F). These data show that a homozygous *dark* mutant background completely reverses the effect of heterozygozity for *Diap1* when tested in the context of apoptotic signaling by *grim* and *hid*. Therefore, in classical genetic terms, *dark* is epistatic to *Diap1* and suggests an order of gene action whereby *dark* functions either downstream or parallel to *Diap1*.

Degeneration of germline cells caused by dysregulation of Diap1 is reversed by dark

We also tested for possible genetic interactions between *Diap1* and *dark* by examining whether germline phenotypes in females that carry two weak *Diap1* alleles *in trans* to one another, $thread^6/thread^8$ ($Diap1^{8/6}$), were modified in a *dark* genetic background (see Materials and methods). As depicted in Table I, $Diap1^{6/8}$ mutant females are not absolutely sterile, but the vast majority (>90%) of these mutants fail to lay eggs when mated to *yw* males. However, when $dark^{CD4}$; $Diap1^{6/8}$ females are scored similarly, egg laying is rescued in more than half of these animals (Table I). To assess directly the reversal of the *Diap1* egg-laying defect by *dark*, we compared ovaries from $Diap1^{6/8}$ flies with ovaries from $dark^{CD4}$; $Diap1^{6/8}$ mutants. $Diap1^{6/8}$ flies have severely stunted and irregular shaped ovaries that appear atrophied, while $dark^{CD4}$; $Diap1^{6/8}$ females produce ovaries that are considerably larger and generally wild-type in appearance. Individual ovarioles dissected 4–5 days post-eclosion are shown in Figure 4. Whereas the vast majority of $Diap1^{6/8}$ late-stage

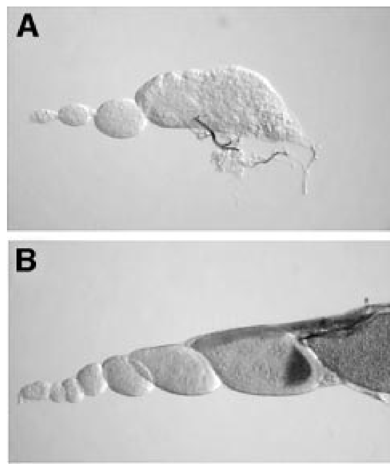

Fig. 4. Ovarian atrophy caused by *Diap1* malfunction is reversed by *dark*. Nomarski color micrographs of *Diap1*$^{6/8}$ (A) and *dark*^{CD4}/*dark*^{CD4}; *Diap1*$^{6/8}$ (B) mutant ovarioles. (**A**) A *Diap1*$^{6/8}$ mutant ovariole that is poorly developed and atrophied. Note that the late egg chamber shows abnormal morphology and signs of degeneration. (**B**) By comparison, a double mutant ovariole shows improved distribution of egg chambers and advanced maturation of the oocyte.

egg chambers exhibit overtly degenerative phenotypes (Figure 4A), most *dark*^{CD4}; *Diap1*$^{6/8}$ egg chambers are normal in morphology and number (Figure 4B). Thus, loss of *dark* function rescues functional ovaries from an otherwise degenerative fate caused by the misregulation of *Diap1*.

dark blocks ectopic cell death in *Diap1* mutant embryos

We next tested whether *dark* might similarly influence phenotypes associated with the complete absence of *Diap1* function. For this purpose, we examined *dark*^{CD4}; *Diap1*^{5} double mutant embryos to determine whether *dark* might rescue defects associated with a null mutation for *Diap1* (i.e. *thread*^{5}/*Diap1*^{5}; see Materials and methods). *Diap1*^{5} mutant animals progress normally through gastrulation but they arrest at the beginning of germ band extension (stage 7) and, within 90 min thereafter, adopt a characteristic morphology reflecting catastrophic events associated with widespread and synchronous apoptosis (Wang *et al*., 1999; this study). The nuclei of most, if not all, cells in *Diap1*^{5} embryos exhibit apoptotic DNA fragmentation detected as extensive TUNEL labeling at a time, in wild-type embryos, that would otherwise correspond to extension of the germ band, stages 7–10 (Wang *et al*., 1999; see Figure 5B). However, in *Diap1* mutant embryos that are also homozygous for *dark*, apoptotic cell death is remarkably attenuated (Figure 5C) and, quite strikingly, the developmental arrest characteristic of single *Diap1* mutants does not occur (Figure 5, compare B and C). Instead, the vast majority of double mutants develop far beyond the embryonic stage at which single *Diap1* mutants arrest. Whereas *Diap1*^{5} mutants catastrophically arrest at stage 7, >90% of *dark*^{CD4}; *Diap1*^{5} embryos show clear signs of normal cell migration and differentiation such that morphological landmarks utilized (e.g. the appearance of the periodic bulges, germ band retraction, stomodeum) for purposes of staging through stage 10 are readily discernible (compare Figure 5A with C). Together, these observations establish that *dark* attenuates inappropriate apoptosis and reverses early morphogenetic arrest caused by the *Diap1* null mutation. Thus, intact *dark* function is required for both ectopic apoptosis and the catastrophic arrest caused by the absence of Diap1. It is worth noting that, while most *dark*^{CD4}; *Diap1*^{5} embryos are spared from an early morphogenetic arrest, limited apoptosis does occur, and these animals are not spared from less severe abnormalities occurring at more advanced stages (compare Figure 5A with C with regard to TUNEL staining and embryo morphology). On rare occasions, we observed a novel class of *dark*^{CD4}; *Diap1*^{5} double mutants as shown in Figure 5D. These occurred at only 5% of the expected frequency and, while they fail to stain significantly for TUNEL, they also appear arrested at stage 7 like their single *Diap1* mutant counterparts. Therefore, suppression of cell death *per se* is not always sufficient for morphogenetic ‘rescue’.

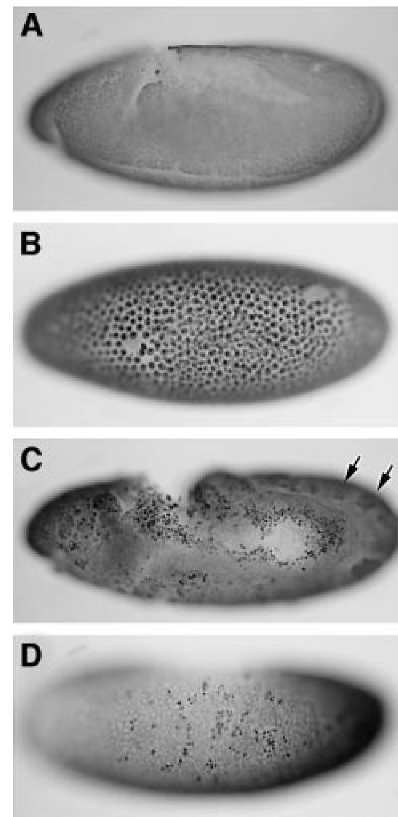

Fig. 5. Loss of *dark* reverses developmental arrest and prevents cell death in *Diap1* embryos. Nomarski micrographs of 4- to 5-h-old embryos labeled by TUNEL. (A) Wild-type, (B) *Diap1*^{5} and (C and D) *dark*^{CD4}; *Diap1*^{5} embryos. (**A**) At, or prior to, the extended germ band stage (stage 9), no programmed cell death occurs in wild-type embryos. (**B**) In contrast, *Diap1*^{5} homozygous embryos of similar chronological age show widespread TUNEL labeling in virtually all cells and exhibit a characteristic ‘blastoderm’-like morphology. (**C**) By comparison, *dark*^{CD4}; *Diap1*^{5} mutant embryos progress considerably further in development (large arrows indicate the periodic bulges seen during stage 10) and are dramatically attenuated for TUNEL labeling. Note that even though this embryo shows germ band retraction, it has some inappropriate TUNEL labeling along with irregularities in embryonic development (i.e. an abnormal hole in the posterior midgut primordium). (**D**) A rare *dark*^{CD4}; *Diap1*^{5} mutant embryo which, though not rescued for the ‘blastoderm’-like morphology, shows TUNEL labeling in only a few cells [compare with (B)].

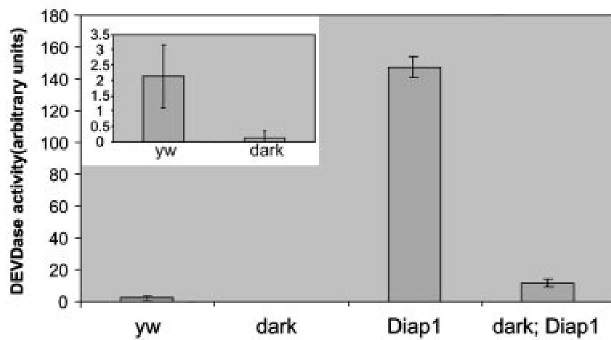

Fig. 6. Caspase 'hyperactivation' triggered by the removal of *Diap1* requires *dark* function. Embryo protein extracts were analyzed for caspase activity by measuring Ac-DEVD-AFC release over time. Extracts derived from wild-type (*yw*), *dark*^{CD4} (dark), TM6/*Diap1*^{5} (Diap1), and *dark*^{CD4}; TM6/*Diap1*^{5} (dark; Diap1) stocks were tested. Note that the inset shows *yw* and *dark* caspase activities, but at a smaller scale for ease of viewing. Each result was verified at least three times, and triplicate samples were recorded from each lysate. $P < 0.005$, two-tailed homoscedastic *t*-test.

dark is required for excessive caspase activity observed in Diap1 embryos

We also examined epistasis between *Diap1* and *dark* at the biochemical level by directly measuring caspase activity from stage 9–10 embryos using a fluorogenic DEVD substrate (Wang *et al*., 1999; see Materials and methods). Compared with wild-type (*yw*) strains, embryonic lysates from populations containing homozygous (stage 9–10) *Diap1*^{5} mutants show highly elevated caspase activity. DEVDase activity in *Diap1*^{5} lysates is elevated by levels ⩾70-fold relative to lysates from control *yw* embryos (Figure 6). Enzymatic 'hyperactivation' in this context is thought to reflect the action of these proteases unrestrained by native inhibitors (Goyal, 2001). In contrast, lysates from *dark*^{CD4}; *Diap1*^{5} embryos show at least 80–90% lower DEVDase activity when compared with *Diap1*^{5} lysates (Figure 6). Thus, a *dark* mutant background prevents 'hyperactivation' of caspase activity that otherwise occurs in the absence of *Diap1* zygotic function. This finding indicates that liberation of caspases from negative regulation is not sufficient to achieve hyperactivation of caspases seen in *Diap1* mutant embryos. To exclude the possibility that some portion of the activities measured in these assays might occur after the lysis step, we mixed *Diap1*^{5} lysates with either wild-type or *dark*^{CD4} lysates and assayed caspase activity from these as well. We detected no synergistic effects indicative of post-lysis amplification of caspase activity in these mixed lysates (data not shown). Therefore, all measureable activity shown in Figure 6 reflects the authentic *in vivo* status in the embryo prior to lysis.

It is worth noting here that lysates from *yw* and *dark* embryos were collected several hours before the onset of PCD (Abrams *et al*., 1993). These basal DEVDase measurements could therefore reflect a low constitutive flow of 'leaky' caspase activity occurring in the complete absence of apoptotic signals. Moreover, since activity in *dark*^{CD4} lysates is considerably lower (~4-fold) than control *yw* lysates, positive input from Dark evidently contributes to 'basal' DEVDase levels (Figure 6).

Discussion

Disruption of caspase–Diap1 interactions by reaper/grim/hid alone is insufficient to induce PCD in vivo

Mutations in *dark* were shown previously to cause a variety of developmental defects, including an enlarged larval CNS (Rodriguez *et al*., 1999). We extend previous observations and establish that, in the absence of *dark* function, cells specified to die actually survive and also differentiate (Figure 1). The embryonic CNS midline glia is a well-studied cell lineage in the fly embryo (Zhou *et al*., 1995; Dong and Jacobs, 1997) and, though we only followed this particular lineage, it is likely that supernumerary cell types in other lineages also persist in *dark* mutants. Several important conclusions derive from our observations. First, the persistence of extra cells excludes trivial explanations for reduced TUNEL labeling in *dark* mutants (e.g. cell death in the absence of TUNEL labeling or redirected cell fates that prevent the specification of PCD) and reinforces models that favor a fundamental role for *dark* in embryonic PCD. Secondly, as previously shown in *C.elegans* (Metzstein *et al*., 1998) and in studies of Reaper interval *Drosophila* mutants (White *et al*., 1994), rescue from PCD can uncover a cryptic differentiation program. Thirdly, and most importantly, the same midline glial cells that fail to die in *dark* mutants also require the induced action of *reaper*, *grim* and *hid* (Zhou *et al*., 1995; Dong and Jacobs, 1997). While it could be argued that extra cells might arise from altered cell division or differentiation, we favor explanations relating to defective PCD since *dark* mutants are already known to exhibit reduced apoptosis in this tissue (Rodriguez *et al*., 1999) and Reaper interval mutants have a similar phenotype in midline glia but exhibit no defects in cell division (Zhou *et al*., 1995; Dong and Jacobs, 1997). Therefore, supernumerary embryonic cells present in *dark*^{CD4} mutants (Figure 1) probably survive because of lack of apoptosis instead of altered cell identity. Experiments in Figure 1 also serve to highlight a requirement for *dark* function in normal PCD when *reaper*, *grim* and/or *hid* are expressed at physiological levels. It is noteworthy that *Diap1* and at least two of the cell death initiators (i.e. *reaper* and *hid*) are expressed in pools of progenitor cells, which give rise to the embryonic midline glia and also play an important role in specifying the death of these cells (Zhou *et al*., 1995, 1997; Wing *et al*., 1998). Thus, although midline glia presumably receive death signals from *reaper* and *hid* leading to the disruption of Diap1–caspase interactions, these cells still fail to die and are even capable of differentiating normally in the absence of *dark* function. This inference supports conclusions from our epistasis studies (discussed below) indicating that a disruption of caspase–Diap1 interactions alone is insufficient for apoptosis, and suggests that *dark* functions as a co-effector of cell death signaling along with *reaper*, *grim* and/or *hid* (see model in Figure 7). These findings are consistent with our previous observations (Rodriguez *et al*., 1999) and the observed induction of *dark* expression upon UV (Zhou *et al*., 1999) or X-ray exposure (Figure 2D). As such, they validate models placing *dark* in the same pathway, downstream or parallel to these apoptotic activators. Finally, it is worth noting

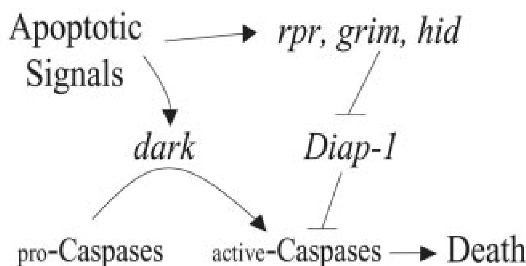

Fig. 7. A 'gas and brake' model for the action of *dark* and *Diap1* in cell death. Studies described here establish that *dark* functions downstream or parallel to the action of *Diap1*. We favor a parallel converging arrangement since, like Apaf-1, Dark acts positively upon apical procaspases while IAPs act negatively upon processed caspases. The model draws upon analogies to the 'gas' and 'brake' controlling forward movement (apoptosis) of a car. In order for PCD to occur, the gas (*dark*) must be engaged and the brake (*Diap1*) must be released in concert. Release of the brakes by *reaper* (*rpr*), *grim* or *hid* will only induce effective apoptosis (movement) if the gas (*dark*) is engaged concurrently by apoptotic stimuli.

that the mammalian ortholog of *dark*, Apaf-1, functions downstream from the point at which mitochondrial factors including cytochrome *c* are released from mitochondria. If the same holds true for the fly protein, it follows that embryonic midline glia (and perhaps other cell types) can survive and differentiate beyond this mitochondrial 'point of no return'.

Additional evidence that PCD requires coordinated *dark*-dependent caspase activation in conjuction with the release of IAP-mediated caspase inhibition comes from our tests of *dark* mutants under conditions of stress that elicit apoptotic responses by the embryonic cell death initiators. Like the Reaper interval mutants (White *et al.*, 1994), we find that *dark* mutants also exhibit profound failures in cell death in response to ionizing radiation (Figure 2). Interestingly, the *dark* mutation itself does not interfere with the radiation induction of *reaper* mRNA (data not shown; Brodsky *et al.*, 2000). We can therefore exclude the possibility that resistance to damage-induced apoptosis evident in irradiated *dark* mutants is caused by a disruption of upstream elements in the signaling pathway. Instead, the results raise the possibility that *dark*, like *reaper* (Brodsky *et al.*, 2000), may function as an important effector of *Drosophila p53*- (Ollmann *et al.*, 2000) mediated apoptosis. In addition, our findings reinforce an apoptotic requirement for *dark* (even when *reaper* is transcriptionally induced) and provide evidence for models where cell death signals do not converge on *Diap1* alone (Figure 7). In future studies, it will be interesting to determine the range of damage signals that can engage *dark* activity, since this locus might also function in a broader range of damage signals beyond those provoked by radiation.

dark is epistatic to Diap1

In flies, *Diap1* is thought to function as a rate-limiting brake on apoptotic cell death (reviewed in Hay 2000; Goyal, 2001). If, however, *Diap1* were the most proximal rate-limiting regulator of apoptosis, then the presence or absence of *dark* function should have no influence on *Diap1*-dependent effects. If, on the other hand, *dark* and *Diap1* exert interdependent functions, then the opposite outcome is predicted. The results from our studies provide consistent and compelling evidence favoring the latter scenario since, wherever tested, the influence of *Diap1* upon apoptotic signaling is heavily dependent upon intact *dark* function. In the $dark^{CD4}$ mutant, for instance, the $Diap1^{5}$ mutation fails to enhance *grim*- and *hid*-induced cell killing in the eye (Figure 3). These experiments examined *Diap1* in the heterozygous condition, but the same outcome is also true when *Diap1* is tested in the homozygous state. Early in embryonic development, *Diap1* homozygotes exhibit catastrophic phenotypes including morphological arrest soon after gastrulation, extensive TUNEL-positive nuclei and hyperactivation of caspases (Wang *et al.*, 1999). Each of these defects is profoundly dependent upon *dark* function since each is dramatically reversed by the *dark* mutation (Figures 5 and 6). In $dark^{CD4}$; $Diap1^{5}$ double mutant embryos, we find no evidence for widespread apoptosis and, in fact, the large majority of these double mutants proceed through stages of embryogenesis well beyond the point at which single *Diap1* mutants arrest. Thus, loss of *dark* not only suppresses apoptotic deaths that would otherwise occur, but definitively reverses a profound morphogenetic arrest that ensues in the absence of *Diap1*. Consequently, homozygosity at *dark* not only prevents the onset of apoptotic markers (i.e. DNA fragmentation and/or caspase activation), but actually preserves cells that are otherwise fated to die when normal checks upon caspases are removed. Rescue from inappropriate apoptosis in this instance is consistent with what we observed in the midline glia (Figure 1) but, rather than preserving cells fated for programmed death, rescue occurs even when signaling from IAP antagonists in the Reaper region is bypassed. We obtained a similar result, with similar implications, in the ovary, where ~90% of heteroallelic $Diap1^{6/8}$ mutants show abnormal degeneration of early staged egg chambers and associated sterility (Figure 4; Table I). Again, loss of *dark* function not only suppresses the pathological effect, but actually reverses these degenerative defects to the extent that half of the $dark^{CD4}$; $Diap1^{6/8}$ double mutant females produce normal egg chambers and are fertile (Figure 4; Table I). Thus, in both the ovary and the embryo, loss of *dark* rescues functional cells from apoptosis caused by misregulated *Diap1*.

Reversal of *Diap1*-dependent defects by *dark* is also evident when we directly assay caspase activity in early embryos. In contrast to the 'hyperactivated' caspase levels detected in *Diap1* single mutant lysates, $Diap1^{5}$; $dark^{CD4}$ lysates show levels of caspase activity that are suppressed >90% relative to *Diap1* mutants (Figure 6). The unusually high caspase activity detected in *Diap1* mutant embryos is thought to reflect the action of these proteolytic enzymes unimpeded by native inhibitors (Wang *et al.*, 1999). However, studies here demonstrate that removing a negative regulator alone is not sufficient to achieve caspase hyperactivation, since *dark* is clearly required for this unrestrained activity. It is worth mentioning that the basal DEVDase activity in double mutants is still somewhat elevated compared with control embryos (Figure 6). This might indicate *dark*-independent caspase activity due to caspase auto-activation in the absence of *Diap1* or, since it is possible that the $dark^{CD4}$ allele used

here is not a complete null, it could reflect hypomorphic *dark* function. Alternatively, since a role for *Diap1* in cytokinesis has not been ruled out (Lisi *et al*., 2000), it is possible that this residual caspase activity ensues because of secondary developmental defects leading to cell death. Nevertheless, these enzymatic data extend our analyses to the biochemical level in a manner fully consistent with our phenotypic studies.

Interestingly, since Apaf-1/Dark adaptor proteins act upon procaspases (Zou *et al*., 1999) while IAPs preferentially inhibit processed caspases (Goyal, 2001), our results further suggest that pre-existing levels of processed caspases in most cells are probably not high enough to achieve an apoptotic threshold. Instead, a positive cell death stimulus from *dark* is required for the unusually high levels of caspase activation seen in $Diap1^{-/-}$ embryos. It is also evident that *Dark*-dependent 'basal' levels of DEVDase are detected in these assays (Figure 6) several hours before the onset of embryonic PCD (Abrams *et al*., 1993), and therefore authentic effector caspase (e.g. DrICE, Dcp-1) activity occurs even in the absence of overt apoptotic signals. These data indicate that constitutive levels of active effector caspases, not derived from autoproteolysis but instead promoted by Apaf-1-like adaptor proteins, may exist in many and perhaps all viable cells.

Taken together, a strict Diap-1–caspase 'liberation' model does not explain sufficiently the evidence described. We show that the action of *dark* is epistatic to that of *Diap1*, demonstrating an order of gene action whereby *Dark* functions either downstream or parallel to *Diap1*. Therefore, simple derepression of caspases via an IAP inhibitory bridge does not account adequately for epistasis between *Diap1* and *dark*. Put another way, our results support the notion that apoptotic cell death *in vivo* results from the simultaneous activation of caspases by *dark* and the derepression of caspases by *reaper*, *grim* and/or *hid*. Accordingly, our findings are inconsistent with models that presume that *Diap1* is the sole effector of *reaper*, *grim* and *hid* and that cells are 'pre-loaded' with sufficient levels of IAP-inhibited processed caspases to achieve cell killing. Instead, we favor a 'gas and brake' model whereby positive input from Apaf-1/Dark adaptors, together with removal of IAP inhibition, drives caspase activation to levels that exceed a threshold necessary for apoptosis (Figure 7).

Materials and methods

Genetics of dark alleles

All crosses and experiments were carried out at 25°C using the previously described $dark^{CD4}$ mutation (Rodriguez *et al*., 1999) derived from a nearby P-element referred to as P1041 or l(2)k11502 (http://www.flybase.net/). The l(2)k11502 strain contains a P-element mapping ~1.3 kb upstream of the *dark* translation initiation site. This P-element (referred to as $dapaf1^{K1}$ in Kanuka *et al*., 1999) was thought to be a lethal allele of the *dark* locus (Zhou *et al*., 1999). However, we find that this insertion, referred to here as $dark^{P1041}$, is at best a weak hypomorphic *dark* allele (Rodriguez *et al*., 1999). For instance, $dark^{P1041}$ shows only slight cell death defects in the embryo and barely detectable defects in adults as compared with $dark^{CD4}$ animals (Kanuka *et al*., 1999; Rodriguez *et al*., 1999; Zhou *et al*., 1999). Secondly, while $dark^{CD4}$ blocks most if not all cells induced by X-ray treatment, $dark^{P1041}$ is indistinguishable from wild-type (data not shown). Thirdly, *in situ* hybridization analysis indicates that $dark^{CD4}$ causes a severe reduction in *dark* mRNA distribution throughout the embryo (data not shown), whereas $dark^{P1041}$ reportedly only disrupts expression in the procephalic head region (Zhou *et al*., 1999). We should note that in studies with $dark^{CD4}$ mutants, we find no evidence for extra photoreceptor cells in the pupal eye (data not shown). Also note that the *yw* strain is used as control here and throughout our studies since it is the parental genotypic backgound for the production of $dark^{CD4}$.

Diap-1 and dark epistatic tests in the retina

For the GMR-*grim* cell killing experiments, females of the genotype *yw*; $dark^{CD4}$, P[GMR-*grim*]-1/$dark^{CD4}$; $Diap1^5$/+ were examined for suppression of induced *grim* killing in the eye as compared with *yw*; P[GMR-*grim*]-1/+; $Diap1^5$/+ females. For GMR-*hid* killing experiments, females of the genotype, *yw*; $dark^{CD4}$, P[GMR-*hid*]-1M/$dark^{CD4}$; $Diap1^5$/+ were compared with *yw*; P[GMR-*hid*]-1M/+; $Diap1^5$/+ females. We were unable to construct the appropriate strains to perform similar experiments with GMR-*reaper* transgenes. All crosses were carried in parallel at 25°C. Micrographs were taken using a Coolpix Nikon digital camera.

Mutant embryo collections and TUNEL staining

Previously, it was demonstrated that maximal TUNEL cell death of $Diap1^5$ null embryos occurs during late stage 9 (Wang *et al*., 1999). Therefore, stage 9–10 embryos were collected and apoptosis assayed using TUNEL labeling. Briefly, embryos were collected for 40 min from adults of the genotype *yw*; $dark^{CD4}$/$dark^{CD4}$; TM6/$Diap1^5$, which previously had been pre-cleared twice, and the embryos were aged for ~4 h. *Diap1* mutant embryos were collected from adults of the genotype *yw*; TM6/$Diap1^5$ in parallel as described above. TUNEL labeling is essentially as in Rodriguez *et al*. (1999). In addition, the 3,3′-diaminobenzidine–peroxide signal was enhanced with nickel chloride and the embryos were pre-cleared using methyl salicylate and viewed using a Zeiss microscope (Patel, 1994).

Caspase activity assays from embryos

For the caspase activity experiments, embryos were collected from either (i) *yw*, (ii) *yw*; TM6/$Diap1^5$ or (iii) *yw*; $dark^{CD4}$; TM6/$Diap1^5$ fly strains. Pools of ~50–100 dechorionated embryos were transferred to an Eppendorf tube with a brush and suspended in 100 µl of buffer A (20 mM HEPES–KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride). Embryos were crushed with a disposable Kontes pestle in a 1.5 ml Eppendorf tube for ~1 min. Cellular debris and lipids were separated from the soluble fraction by a 15 min spin at 15 000 *g* at 4°C, and the protein concentrations were measured and normalized by addition of buffer A. A 5 µg aliquot of protein extract was incubated with 50 µM AcDEVD-AFC (Calbiochem) substrate in a final volume of 20 µl in a 384 microtiter plate. Fluorescence was monitored over time with excitation at 360 nm and emission at 465 nm in a SpectraFluor Plus plate reader (Tecan).

Fertility assays and ovary dissections

Since the molecular nature of the $thread^8$ ($Diap1^8$) mutation is still undefined, we wanted to safeguard against the possibility that it might represent a mutation at a locus other than *Diap1*. Therefore, we tested the $Diap1^8$ strain for non-complementation against a deletion that removes the *Diap1* gene, the $Diap1^5$ mutation, and also tested for dominant suppression or enhancement of GMR-*hid*-1M and P[GMR-*reaper*]-M (GMR-*reaper*) cell killing phenotypes in the eye. While we find that $Diap1^8$ is lethal *in trans* to the *Diap1* deficiency and $Diap1^5$, we fail to see any modification of cell killing of GMR-*hid*-1M and GMR-*reaper* (data not shown), suggesting that it might represent a very weak *Diap1* mutation. The $Diap1^6$ (also known as th^{6B}) allele has a C412Y missense mutation within the RING domain of *Diap1* and is believed to represent a type 1 gain-of-function mutation because it enhances induced cell killing by *grim* and *reaper* but not *hid* (Lisi *et al*., 2000). Adult females of the appropriate genotype were obtained from crossing $Diap1^6$/TM6C to $Diap1^8$/TM6C flies. $Diap1^6$/$Diap1^8$ transheterozygous flies were selected based on the absence of the dominant balancer. Flies of the genotype CyO/$dark^{CD4}$; TM6/$Diap1^6$ were mated to CyO/$dark^{CD4}$; TM6/$Diap1^8$ in order to obtain *dark* homozygous *Diap1* transheterozygous flies. Single females of the appropriate genotype were mated to *yw* males and monitored for egg laying over the course of 1–2 weeks. Ovary dissections were carried out in phosphate-buffered saline and stained for several hours in FeNap staining buffer (Ashburner, 1989).

Immunohistochemistry

Immunohistochemistry was performed as described in Patel (1994). β-galactosidase was detected using a mouse monoclonal antibody (Promega) followed by detection with anti-horseradish peroxidase-conjugated secondary antibodies using the Vectastain Elite Kit (Vector Labs) (Zhou *et al*., 1995).

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