Mitochondrial Disruption in *Drosophila* Apoptosis

Eltyeb Abdelwahid,[1] Takakazu Yokokura,[1] Ronald J. Krieser,[1] Sujatha Balasundaram,[1] William H. Fowle,[2] and Kristin White[1,*]

[1] Cutaneous Biology Research Center, Massachusetts General Hospital/Harvard Medical School, Building 149, 13th Street, Charlestown, MA 02129, USA
[2] Electron Microscopy Center, Northeastern University, Boston, MA 02115, USA
*Correspondence: kristin.white@cbrc2.mgh.harvard.edu
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SUMMARY

Mitochondrial disruption is a conserved aspect of apoptosis, seen in many species from mammals to nematodes. Despite significant conservation of other elements of the apoptotic pathway in *Drosophila*, a broad role for mitochondrial changes in apoptosis in flies remains unconfirmed. Here, we show that *Drosophila* mitochondria become permeable in response to the expression of Reaper and Hid, endogenous regulators of developmental apoptosis. Caspase activation in the absence of Reaper and Hid is not sufficient to permeabilize mitochondria, but caspases play a role in Reaper- and Hid-induced mitochondrial changes. Reaper and Hid rapidly localize to mitochondria, resulting in changes in mitochondrial ultrastructure. The dynamin-related protein, Drp1, is important for Reaper- and DNA-damage-induced mitochondrial disruption. Significantly, we show that inhibition of Reaper or Hid mitochondrial localization or inhibition of Drp1 significantly inhibits apoptosis, indicating a role for mitochondrial disruption in fly apoptosis.

INTRODUCTION

Mitochondria are thought to play multiple roles in apoptosis in many organisms (Kroemer and Reed, 2000). Most well studied is the contribution of released mitochondrial factors such as Cytochrome *c* (Cyt *c*) to caspase activation (Li et al., 1997). In mammalian systems, Cyt *c* is released on mitochondrial outer membrane permeabilization. This release is regulated by the activities of the Bcl-2 family of proteins (Danial and Korsmeyer, 2004). Cyt *c* binds to Apaf-1, promoting the activation of caspase 9 (Li et al., 1997). Other proapoptotic factors are also released from mitochondria, including AIF, SMAC/Diablo, and Omi/HTRA2 (Danial and Korsmeyer, 2004). Mitochondria also serve as docking sites for components of the apoptotic machinery. Notably, in *C. elegans*, the Ced-4/Ced-9 complex is found at the mitochondria (Chen et al., 2000). On induction of apoptosis, the Egl-1 protein displaces Ced-4 from Ced-9, allowing Ced-4 to interact with the Ced-3 caspase, promoting its activity (Conradt and Horvitz, 1998).

Mitochondrial fission and fusion may also play a role in apoptosis. Recent work shows that Egl-1 promotes mitochondrial fragmentation and apoptosis (Jagasia et al., 2005). Mitochondrial fragmentation also contributes to cell death in yeast (Fannjiang et al., 2004). In both of these models, the dynamin-related protein, Drp1, normally involved in mitochondrial fission, functions in mitochondrial fragmentation in apoptotic cells. In mammalian systems, Drp1-mediated mitochondrial fragmentation contributes to apoptosis, and Drp1 colocalizes with the proapoptotic Bcl-2 family member Bax (Frank et al., 2001; Karbowski et al., 2002).

Despite conservation of the majority of the apoptotic pathway in *Drosophila*, the role of mitochondria in apoptosis is less well understood. Developmental apoptosis in the fly requires the function of the *reaper* (*rpr*), *grim*, and *hid* (RHG) genes (Bangs and White, 2000). A role for the mitochondria in the apoptotic process is suggested by the finding that Rpr, Grim, and Hid are found to localize to mitochondria (Haining et al., 1999; Claveria et al., 2002; Olson et al., 2003). Mitochondrial localization is essential for full killing activity of Rpr and Grim (Claveria et al., 2002; Olson et al., 2003). However, considerable data suggest that the proapoptotic activity of these proteins depends in large part on their ability to bind to and inhibit *Drosophila* Inhibitor of Apoptosis Protein 1 (DIAP1) through an N-terminal IAP-binding motif (IBM) (Kornbluth and White, 2005). In addition, there is considerable evidence that Cyt *c*, the most well-characterized proapoptotic mitochondrial factor, does not play a role in *Drosophila* apoptosis. Cyt *c* is not released in drug- or UV-induced apoptosis in fly cells (Dorstyn et al., 2002; Zimmermann et al., 2002; Means et al., 2006). However, previous studies have shown a change in the exposure of a Cyt *c* epitope in response to caspase activation downstream of Rpr or Grim expression, but this was not associated with release of Cyt *c* (Varkey et al., 1999). Also, the *Drosophila* apoptosome appears to assemble without Cyt *c* (Yu et al., 2006). In vivo data support a role for Cyt *c* in caspase activation during spermatogenesis and timely death of interommatidial cells (Arama et al., 2003, 2006; Huh et al., 2004; Mendes et al., 2006). Thus, Cyt *c* does not appear to be required for the majority of developmental apoptosis in flies, but a more general

role for mitochondrial changes cannot be ruled out. Very recently, *Drosophila* Omi/HTRA2 has been implicated in apoptosis (Igaki et al., 2007).

To address this issue, we performed a careful analysis of the effects of Rpr and Hid on mitochondria, assessing both altered mitochondrial permeability and changes in mitochondrial ultrastructure. We found that Cyt *c* was released in cells expressing Rpr and Hid, but confirmed that Cyt *c* release did not play a role in caspase activation. We also found that Rpr and Hid caused changes in mitochondrial ultrastructure. We further showed that the mitochondrial fission mediator Drp1 is important for mitochondrial changes in response to Rpr expression and ionizing radiation. Most importantly, inhibition of Drp1 resulted in a significant inhibition of mitochondrial disruption and apoptosis. Taken together, these data suggest that Rpr and Hid killing involves changes in the mitochondria and suggest a conserved role for mitochondria between flies and mammals.

RESULTS

Rpr and Hid Rapidly Permeabilize the Mitochondria and Release Cyt *c*

Because the endogenous apoptosis regulators Rpr and Hid induce apoptosis very rapidly in vivo (Grether et al., 1995; White et al., 1996), we used an inducible transient transfection system to observe early changes in the mitochondria of apoptotic cells. We first assayed the effect of Rpr and Hid on mitochondrial permeability by analyzing the distribution of the mitochondrial protein Cyt *c* by immunohistochemistry. Cyt *c* distribution was assessed with an anti-mouse Cyt *c* antibody that recognizes at least one form of *Drosophila* Cyt *c* (see Figure S1 available with this article online).

At 90 min after induction of Rpr or Hid expression, many cells exhibited a diffuse cytoplasmic distribution of Cyt *c* (Figures 1E and 1H) and apoptotic morphology (Figures 1D and 1G). Rpr expression resulted in a more complete release of Cyt *c*, with approximately 30% of transfected cells showing cytoplasmic Cyt *c*. An additional 15% showed some cytoplasmic Cyt *c* (partial release) after 90 min (Figure 1U). Thus, almost half of the Rpr-expressing cells release Cyt *c*. This is also true for Hid-expressing cells, although a higher percentage of Hid-expressing cells showed only partial Cyt *c* release. These data indicate that Rpr and Hid promote the rapid permeabilization of the mitochondria and consequent release of Cyt *c*. However, it is important to note that Cyt *c* does not contribute to Rpr- or Hid-induced apoptosis in our system (Figure S1).

We also assayed Cyt *c* release with a fluorescence-activated cell sorting (FACS) assay (Waterhouse and Trapani, 2003). In this assay, cells are treated with digitonin to permeabilize the plasma membrane but not the mitochondrial membrane. Any Cyt *c* that is not confined to the mitochondria is allowed to wash out of the cell. Cells are stained for Cyt *c*, and the assay measures the amount of Cyt *c* remaining in the mitochondria of each cell. In cells

where the mitochondria are intact, Cyt *c* staining is high. In cells where the mitochondria have become permeable and Cyt *c* is mainly cytoplasmic, the level of Cyt *c* fluorescence is lower. We selected this assay over the more traditional cell fractionation as the percentage of total cells where Cyt *c* release is detected by immunohistochemistry is a relatively small proportion of the total cells in the culture. We, like others, were unable to detect cytoplasmic Cyt *c* in *Drosophila* cells by fractionation (data not shown) (Varkey et al., 1999; Zimmermann et al., 2002; Means et al., 2006). By this FACS assay, the proportion of cells retaining high Cyt *c* was significantly reduced on Rpr or Hid expression, with Rpr expression resulting in more complete permeabilization of mitochondria (Figures 1F and 1I). These results are consistent with our histological analysis.

The Role of Caspase Activation in Mitochondrial Permeabilization

Many studies have shown that Rpr and Hid activate apoptosis, at least in part, by binding and inhibiting DIAP1, resulting in caspase activation (reviewed in Goyal, 2001). If caspase activation is sufficient to permeabilize the mitochondria to Cyt *c*, then other apoptosis-inducing agents such as Actinomycin D and UV should also result in Cyt *c* release. We assayed for Cyt *c* release in response to these treatments, at a time when levels of caspase activity were at least as high as that induced by Rpr and Hid (Figure 1S). Unlike Rpr and Hid expression, neither Actinomycin D treatment nor UV light exposure resulted in Cyt *c* release (Figures 1J–1O). To eliminate the possibility that Cyt *c* was released transiently at an earlier stage of apoptosis, we examined Cyt *c* release at 3, 6, and 8 hr after Actinomycin D treatment and at 4, 8, 12, and 16 hr after UV exposure. Cyt *c* release was not detected at any of these time points (data not shown). These data indicate that permeabilization of mitochondria is not an inevitable downstream event of caspase activation in S2 cells and suggest that Rpr and Hid have additional activities in the dying cell causing this permeabilization.

To determine whether Rpr- and Hid-induced mitochondrial permeabilization depends on inhibition of DIAP1, we examined mitochondrial permeabilization in DIAP1-depleted cells. Knockdown of DIAP1 by RNA interference (RNAi) did not result in Cyt *c* release, despite extremely high levels of caspase activity (Figures 1P–1S). Thus, the proapoptotic functions of Rpr and Hid are not restricted to DIAP1 inhibition, but also include an activity that promotes mitochondrial permeabilization.

Although caspase activation itself was not sufficient for mitochondrial permeabilization, it could play a role in Rpr- or Hid-induced mitochondrial permeabilization. To test this possibility, we induced Rpr or Hid expression in the presence of the pan-caspase inhibitor zVAD-FMK and examined Cyt *c* release by confocal microscopy, as well as FACS. These assays indicated that permeabilization of the mitochondria to Cyt *c* was inhibited in the presence of zVAD-FMK (Figures 1T and 1U and data not shown). Cyt *c* remained punctate in many of the transfected cells. In others, it was more diffuse than in controls (compare
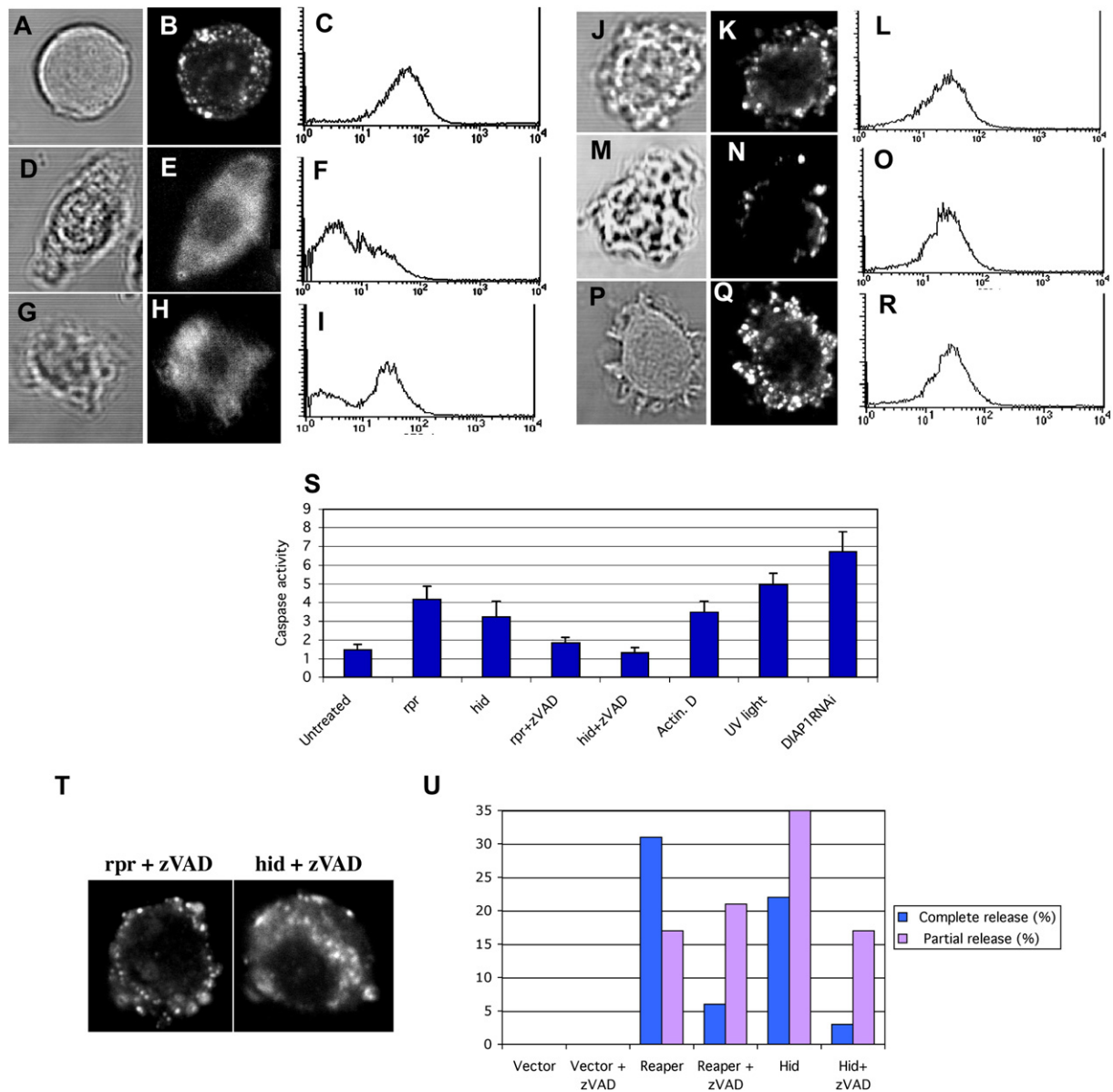
Figure 1. Mitochondria Are Rapidly Permeabilized in Response to Rpr or Hid Expression

(A–R) Immunofluorescence and fluorescence-activated cell sorting (FACS) analysis of Cyt *c* release in *Drosophila* S2 cells in response to apoptotic stimuli. When observed with Nomarski optics (A, D, G, J, M, and P), the dying cells adopt typical features of apoptosis. Untreated cells (A–C) and empty-vector transfected cells (Figure 4B) showed punctate mitochondrial localization of Cyt *c*. Both Hid (D–F) and Rpr (G–I) expression resulted in cytoplasmic Cyt *c* (E and H). In contrast to Rpr and Hid, cells killed by UV light (J–L), Actinomycin-D (M–O), and DIAP1 RNAi (P–R) did not cause Cyt *c* release from the mitochondria (K, N, and Q) despite characteristic apoptotic morphology. FACS analysis of mitochondrial permeability to Cyt *c* confirmed the immunohistochemical findings. Untreated cells (C) as well as empty-vector-transfected cells (Figure 4D) were used as controls. Cells killed by Hid (F) and Rpr (I) showed significant mitochondrial permeability to Cyt *c*, as indicated by a peak with lower Cyt *c* staining after permeabilization. Apoptosis induced by UV light (L), Actinomycin-D (O), and DIAP1 RNAi (R) did not result in mitochondrial permeability to Cyt *c*.

(S) Caspase activity of cell lysates from cells treated with apoptosis inducers. All apoptotic stimuli resulted in increased DEVDase activity (mean ± SD).

(T) The pan-caspase inhibitor zVAD-FMK partially suppressed Cyt *c* release in Rpr and Hid expressing cells. Caspase inhibition increased the number of cells with partial Cyt *c* release, and some mitochondrial Cyt *c* staining in these cells looked more diffuse.

(U) Quantitative analysis of immunohistochemistry as shown in (E), (H), and (T). At least 100 transfected cells were scored for complete or partial Cyt *c* release on expression of Rpr or Hid, with or without zVAD-FMK. Caspase inhibition by zVAD-FMK caused significant inhibition of Cyt *c* release in both Rpr- and Hid-expressing cells. Confocal images were taken at 40× magnification, zoom 8.

Figure 1B with Figure 1T). Taken together, this suggests that permeabilization of the mitochondria is both Rpr or Hid dependent and caspase dependent.

To determine whether mitochondrial membrane potential is lost in apoptotic *Drosophila* cells, we used the potentiometric dye JC-1, which accumulates in polarized
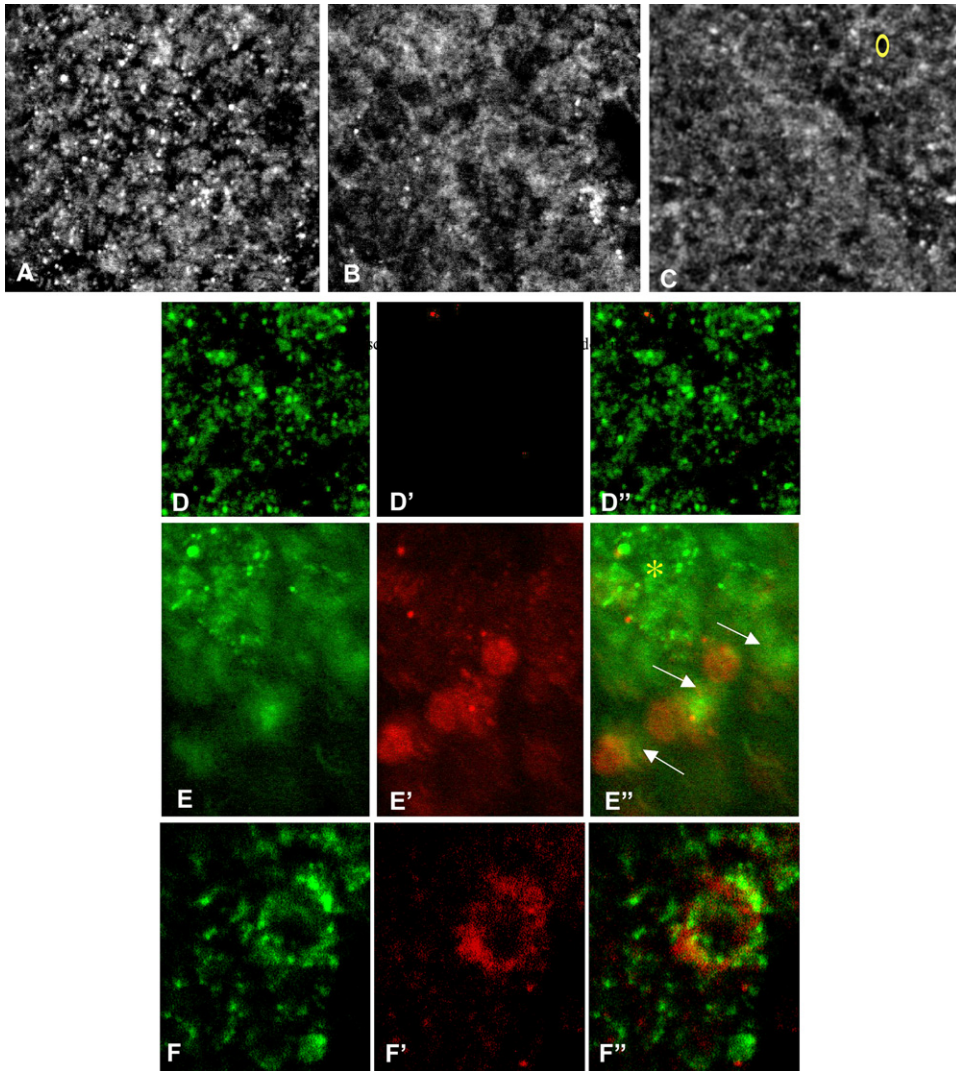
Figure 2. Rpr or Hid Expression in Embryos Promotes Permeabilization of Mitochondria to Cyt *c*

Embryos stained with anti-Cyt *c* ([A]–[C] and green in [D]–[F]) and antiactive caspase 3 (red in [D]–[F]). Ectopic expression of Hid (B) and Rpr (C) disrupted the punctate pattern of Cyt *c* observed in heat-shocked wild-type embryos (A), as shown in these images of the surface epithelium of 6–8 hr old *hs-rpr*, *hs-hid*, and *yw* embryos 90 min after heat shock. For size orientation, the area of a single nucleus has been indicated in (C). Rpr expression resulted in more diffuse Cyt *c* staining than Hid expression. (E) Activated caspase 3 (red) and Cyt *c* (green) localization assessed by immunofluorescence 5 hr after X irradiation of 3–4 hr old embryos. Nonirradiated embryos at similar ages were used as controls (D). Cyt *c* was punctate in activated caspase-3-negative cells (yellow asterisks in [E″]) but was released in activated caspase-3-positive cells (white arrows in [E″]). (F) The release of Cyt *c* in response to X irradiation is dependent on genes in the H99 region. Homozygous H99 embryos contained a small number of cells positive for active caspase at 10 hr after irradiation (F′). Unlike in the wild-type, these cells displayed punctate Cyt *c* staining. Caspase staining was also more punctate. (A)–(D) are at 40× magnification, zoom 4. (E)–(F) are at 40× magnification, zoom 8.

mitochondria (Smiley et al., 1991). In S2 cells transfected with empty vector, the majority of the mitochondria are polarized and fluoresce red (Figure S2). In approximately one-third of Rpr- and Hid-expressing cells, there is significant green fluorescence after 90 min of expression, demonstrating a significant depolarization of mitochondria. This depolarization is mostly caspase dependent. We also found that treatment of cells with Actinomycin D or DIAP1 dsRNA results in the appearance of depolarized mitochondria. Again, this is partially caspase dependent. These data demonstrate that permeabilization of the

mitochondrial outer membrane to Cyt *c* is not a result of depolarization, as both Actinomycin D and DIAP1 dsRNA kill cells and depolarize mitochondria without releasing Cyt *c*.

Mitochondrial Permeabilization Occurs in Dying Cells In Vivo

If Cyt *c* release is a widespread consequence of Rpr and Hid expression, we should be able to detect it in intact animals. We found that expression of Rpr or Hid in embryos from a heat-shock-inducible transgene rapidly disrupted
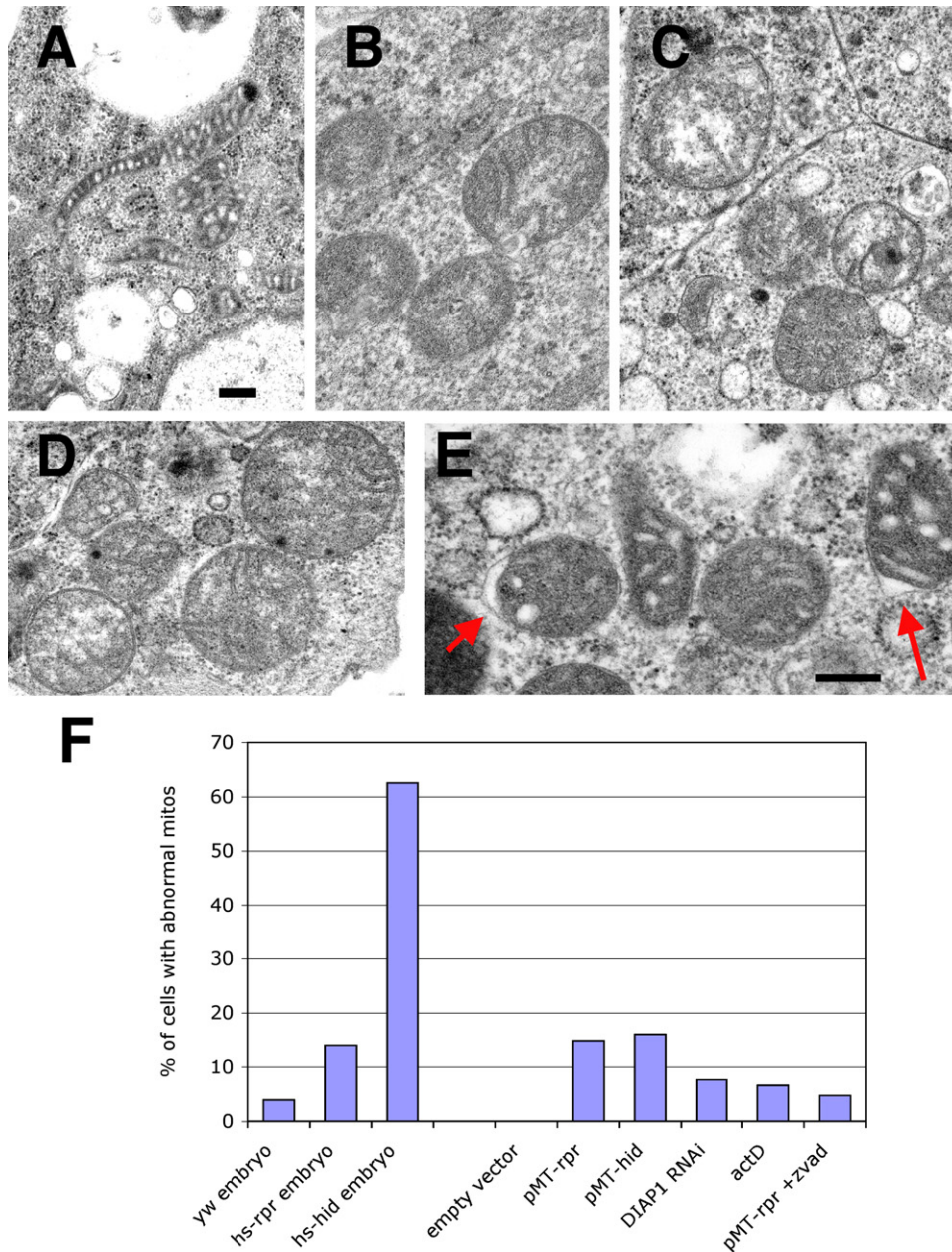
Figure 3. Rpr and Hid Induce Mitochondrial Disruption in a Caspase-Dependent Manner

Transmission electron micrographs of mitochondria in cells induced to die by various stimuli. Mitochondria of S2 cells transfected with empty vector (A) had normal ultrastructure with a dense matrix and intact cristae, with a rather elongated form. Expression of Hid (B) or Rpr (D) for 90 min resulted in mitochondria that appeared swollen and rounded and had a less dense matrix. In embryos, induction of hs-hid also resulted in swollen mitochondria with a less dense matrix (C). In S2 cells, Rpr and Hid expression also caused bulging of the outer membrane of mitochondria (arrows in [E]). All scale bars represent 200 nm. The scale bar in (A) also applies to (B), (C), and (D). Quantification of the percent of cells with abnormal mitochondria is shown in (F).

the punctate pattern of Cyt *c* detected in control embryos (Figures 2A–2C).

To rule out the possibility that mitochondrial permeabilization was an artifact of Rpr or Hid overexpression, we examined Cyt *c* distribution during DNA-damage-induced apoptosis in vivo. After irradiation, diffuse Cyt *c* staining was detected in cells staining positively for active caspase (Figures 2E–2E″). Cyt *c* staining remained punctate in

caspase-positive cells in irradiated H99 homozygous embryos (Figures 2F–2F″). These embryos are very resistant to X-ray-induced apoptosis, due to deletion of Rpr, Hid, and Grim (White et al., 1994). However, the rare apoptotic cells in H99 embryos had punctate Cyt *c* staining. These data demonstrate that mitochondrial permeabilization in dying cells requires the activity of the genes in the H99 region.
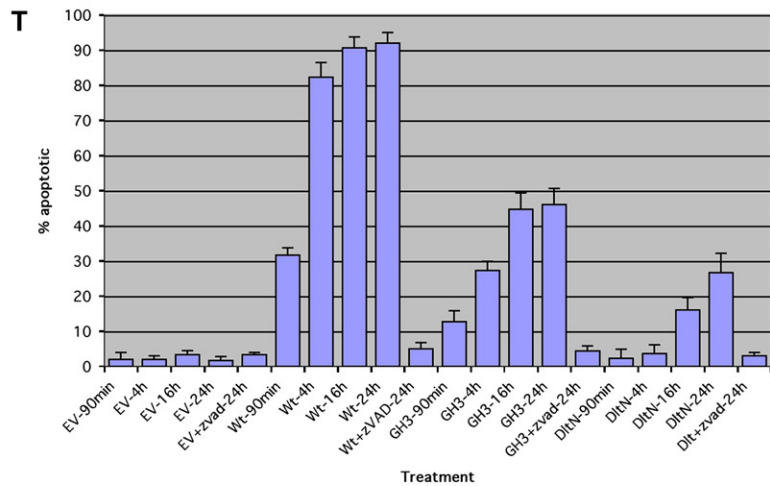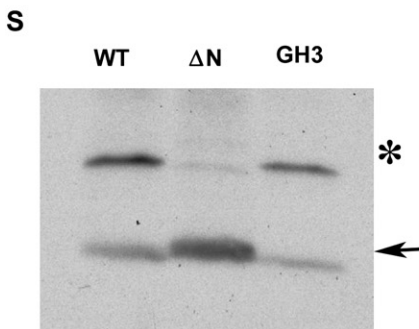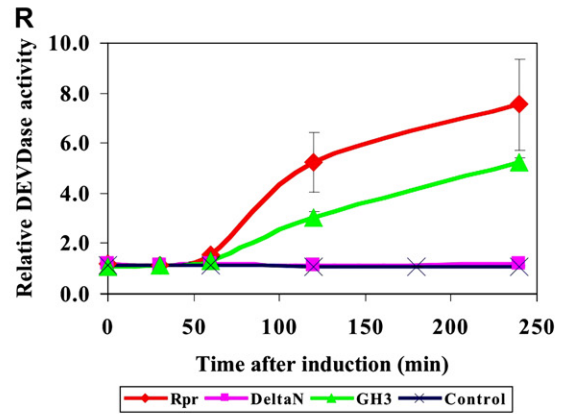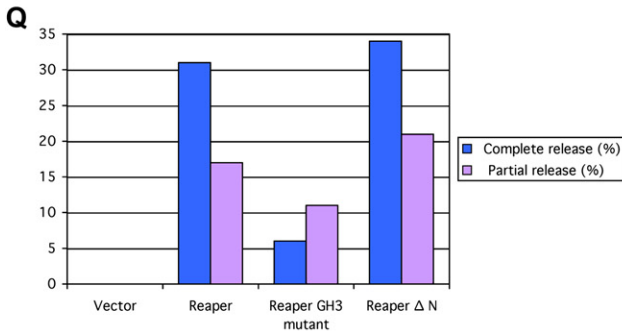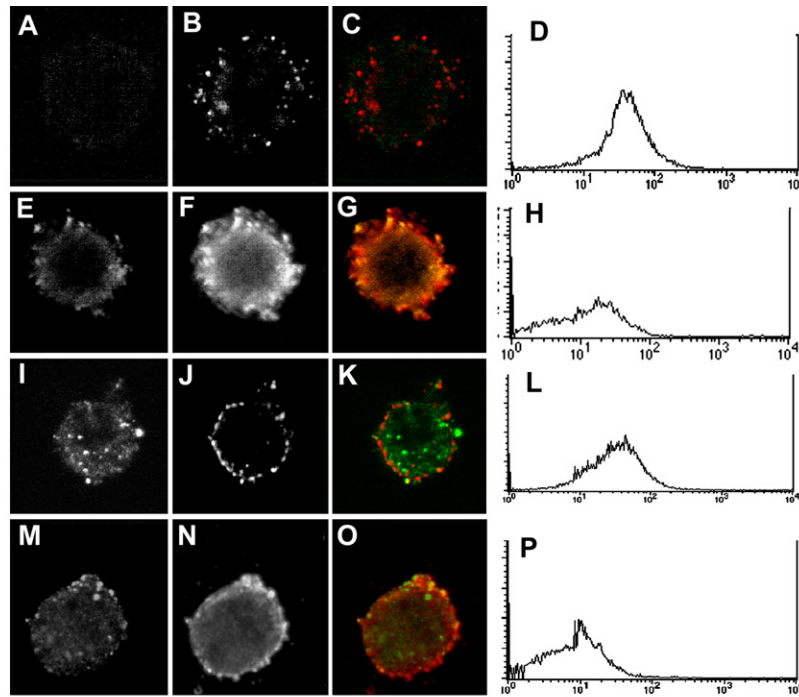
Rpr and Hid Disrupt Mitochondria

We examined the ultrastructure of the mitochondria after Rpr or Hid expression to determine if permeabilization was associated with detectable morphological changes. We found that Rpr or Hid expression resulted in alterations in mitochondrial morphology (Figure 3). In control cells, mitochondria are elongated with a dense matrix and intact cristae (Figure 3A). On Rpr or Hid expression, approximately 15% of cells show altered mitochondrial morphology. This represented about 30%–50% of transfected cells. These alterations range from mitochondrial swelling with a rounder shape, less dense matrix, and cristae that appear broken (Figures 3B and 3D) to less dramatically altered mitochondria, including those that showed a bulging of the outer mitochondrial membrane (Figure 3E). We see similar changes in the mitochondria of embryos after Hid or Rpr expression from a heat-shock promoter (Figures 3C and 3F).

To determine whether the morphological change in mitochondria is a common feature of apoptotic cells, we examined the ultrastructure of mitochondria in S2 cells after treatment with Actinomycin D or DIAP1 RNAi. Loss of DIAP1 and Actinomycin D treatment resulted in some alteration of mitochondrial morphology, with 6%–7% of treated cells showing mitochondrial alterations (Figure 3F). These data demonstrate that structural disruption and permeabilization of mitochondria are correlated, in the sense that Rpr or Hid resulted in both of these mitochondrial changes, while caspase activation alone does not promote either change to the same extent. However, we also found that caspase inhibition blocked Rpr- and Hid-induced changes in mitochondrial structure (Figure 3F and data not shown). Taken together with the requirement for caspase activity in mitochondrial permeabilization, this suggests that Rpr and Hid acting in concert with caspase activation results in mitochondrial disruption and Cyt c release.

Mitochondrial Localization of Rpr and Hid Is Required for Mitochondrial Permeabilization

Rpr and Hid have both been shown to localize to mitochondria: Rpr through an amphipathic helix called the GH3 domain and Hid through a C-terminal domain (Haining et al., 1999; Olson et al., 2003). To determine if localization is required for permeabilization, and whether mitochondrial permeabilization contributes to apoptosis, we generated inducible expression constructs for mutant forms of Rpr (Rpr GH3 mut) and Hid (HidΔC20) that do not localize to mitochondria.

As previously reported, wild-type Rpr was partially localized to mitochondria (Figures 4E–4H and Table S1). In cells where the mitochondria had become permeable to Cyt c, the distribution of Rpr became more diffuse (Figures 4E–4H and 4Q). This suggests that Rpr is released from the mitochondria as cells die. In contrast, the Rpr GH3 mutant protein was localized to punctate structures within the cell that did not overlap with Cyt c staining. This mutant form of Rpr did not cause mitochondrial permeabilization to Cyt c (Figures 4I–4L and 4Q and Table S1).

The Rpr GH3 mutant also induced less apoptosis than the wild-type protein. When protein expression levels were equalized (Figure 4S), expression of the GH3 mutant resulted in a lower level of caspase activation than wild-type Rpr (Figure 4R). Even at later time points, apoptosis induced by the GH3 mutant was much lower than that induced by wild-type Rpr (Figure 4T). This suggests that mitochondrial localization of Rpr contributes to apoptosis in these cells.

Hid has been shown to localize to mitochondria when expressed in mammalian cells (Haining et al., 1999). In S2 cells, we found that wild-type Hid, like Rpr, partially colocalized with Cyt c and became more diffuse as the mitochondria became permeable to Cyt c (Figures 5A–5D and Table S1). A Hid mutant lacking the C-terminal mitochondrial localization domain (HidΔC20) was distributed in a punctate manner that did not colocalize with Cyt c and resulted in less Cyt c release (Figures 5E–5H and 5I and Table S1). HidΔC20 was less stable than wild-type Hid, but even when protein expression levels were equalized, HidΔC20 activated caspases much more poorly than wild-type Hid (Figures 5J and 5K). Taken together, these data suggest that mitochondrial localization is essential for full caspase activation by Hid as well as by Rpr.

Figure 4. The GH3 Domain of Rpr Is Required for Mitochondrial Localization, Cyt *c* Release, and Caspase Activation in S2 Cells

(A–P) Immunofluorescence and FACS analysis of Cyt *c* release in S2 cells transfected with empty vector (A–D), wild-type Rpr (E–H), Rpr GH3 (I–L), and Rpr ΔN (M–P). All Rpr constructs were C-terminally tagged with HA. The cells were stained with anti-HA ([A], [E], [I], and [M]; green in [C], [G], [K], and [O]) and anti-Cyt *c* antibodies ([B], [F], [J], and [N]; red in [C], [G], [K], and [O]). Single confocal sections are shown. Wild-type Rpr was partially localized to the mitochondria and caused Cyt *c* release when expressed (E–H). Both Rpr and Cyt *c* became diffuse in dying cells. The Rpr GH3 mutation (I–L) blocked mitochondrial localization, Cyt *c* release, and mitochondrial permeability to Cyt *c*. In contrast, Rpr ΔN (M–P) was localized to the mitochondria and resulted in Cyt *c* release and permeable mitochondria.

(Q) Quantitative analysis of immunohistochemical detection experiments shown above.

(R) Timeline of DEVDase activation in response to expression of wild-type and mutant Rpr. Rpr ΔN did not induce apoptosis at short times after expression, while the GH3 mutant was less effective than wild-type Rpr in inducing caspase activation.

(S) Anti-HA blot showing expression levels of wild-type, Rpr ΔN, and Rpr GH3 after 2 hr induction (arrow, full-length protein; asterisk, probable mono-ubiquitinated form). Wild-type and Rpr GH3 mutant are expressed at approximately the same levels, while ΔN is present at higher levels and is only ubiquitinated at low levels.

(T) Viability assay indicating the percentages of transfected cells with apoptotic morphology, with or without zVAD-FMK (mean ± SD). Cells with apoptotic morphology were counted 90 min, 4 hr, 16 hr, and 24 hr after induction. Wild-type Rpr causes rapid cell killing; ΔN Rpr kills cells more slowly. Caspase inhibition with zVAD-FMK inhibits apoptosis induced by all forms of Rpr. Confocal images were taken at 40× magnification, zoom 8.
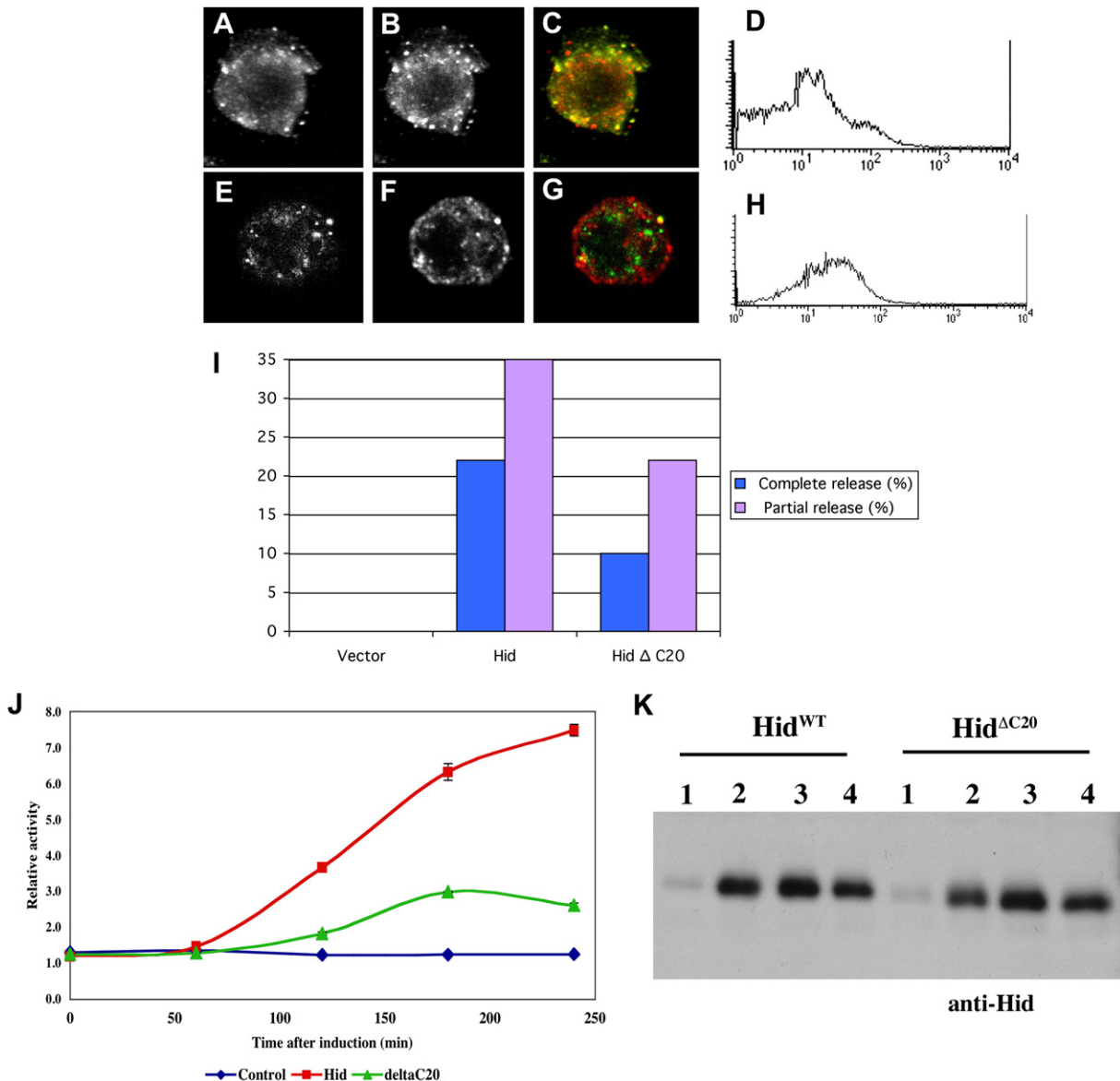
Figure 5. The Hid C20 Domain Is Required for Hid Mitochondrial Localization, Cyt *c* Release, and Caspase Activation in S2 Cells

Immunofluorescence and FACS analysis of Cyt *c* release in S2 cells transfected with wild-type Hid (A–D) and Hid ΔC20 (E–H). Hid was localized to the mitochondria and caused Cyt *c* release and mitochondrial permeability when expressed (A–D). Hid ΔC20 inhibited Hid mitochondrial localization, Cyt *c* release, and mitochondrial permeability (E–H). Control cells transfected with empty vector were shown in Figures 4A–4C. (I) Quantitative analysis of transfected cells that completely or partially released Cyt *c* on expression of wild-type Hid and Hid ΔC20. Wild-type Hid caused release of Cyt *c* into the cytoplasm; however, Cyt *c* release was significantly blocked by Hid ΔC20 (I). (J) Hid ΔC20 also resulted in significantly less DEVDase activation than wild-type Hid, even though it is expressed at slightly higher levels (K). Confocal images were taken at 40× magnification, zoom 8.

RprΔN Permeabilizes Mitochondria and Kills Cells

If permeabilization of mitochondria is sufficient to activate apoptosis, a mutant form of Rpr lacking the N-terminal IBM (RprΔN) should still kill cells. As expected, we found that RprΔN localized to mitochondria and permeabilized the mitochondria to Cyt *c* (Figures 4M–4P and 4Q). Expression of this mutant did not increase caspase activation in the 4 hr time span of our initial assay (Figure 4R). However, in longer-term assays, RprΔN expression did result in significant caspase-dependent apoptosis (Fig-

ure 4T). This demonstrates that Rpr has two killing activities: a rapid inhibition of DIAP1, and a slower activity, which is independent of the IBM. This confirms previous findings that N-terminally truncated Rpr is sufficient to induce apoptosis (Wing et al., 1998; Chen et al., 2004).

Drp1-Dependent Mitochondrial Disruption Is Required for Apoptosis

The dynamin-related protein Drp1 has been implicated in mitochondrial disruption during cell death in mammals,

nematodes, and yeast (Frank et al., 2001; Fannjiang et al., 2004; Jagasia et al., 2005). To test if Drp1 was also involved in mitochondrial disruption in flies, we knocked down the protein with dsRNA. To facilitate visualization of mitochondria, we used a mitochondrially targeted EYFP (mito-GFP) (LaJeunesse et al., 2004). This construct contains a mitochondrial targeting sequence from subunit VIII of Cyt *c* oxidase, localizing GFP to the mitochondrial matrix (Rizzuto et al., 1995).

We found that about 50% of S2 cells treated with dsRNA to Drp1 for 36 hr showed enlarged reticular mito-GFP staining, in contrast to the more punctate staining seen in cells treated with a control dsRNA (Figures 6E and 6F). This is consistent with a role for Drp1 in normal mitochondrial fission and resembles the phenotypes seen on expressing a dominant-negative Drp1 in *C. elegans* and mammalian cells (Frank et al., 2001; Jagasia et al., 2005) and in Drp1 mutants in flies (Verstreken et al., 2005). This altered mitochondrial phenotype suggests that Drp1 protein levels have been successfully reduced by RNAi. Longer treatment with Drp-1 RNAi resulted in a single large clump of mitochondria in each cell (data not shown). We chose to assay the effects of Drp1 at shorter times to avoid possible effects of this dramatic alteration in mitochondria on cell proliferation and viability.

We assessed the role of Drp1 in mitochondrial permeabilization in response to Rpr expression by staining cells with anti-Cyt *c* (Figures 6A–6D) or visualizing mito-GFP (Figures 6E–6J). In response to Rpr expression, mito-GFP rapidly becomes undetectable. We found that Drp1 RNAi prevented the appearance of diffuse Cyt *c* or loss of mito-GFP in more than half of the apoptotic cells (Figure 6K). This suggests that this component of the mitochondrial fission machinery contributes to the disruption of the mitochondria in response to Rpr. Strikingly, we found that Drp1 RNAi significantly inhibited Rpr-induced apoptosis (Figure 6L). This provides important evidence that mitochondrial disruption contributes to apoptosis in these cells. Cell killing by ΔN-Rpr is also suppressed by Drp1 knock down (Figure 6M).

We also examined the role of Drp1 in mitochondrial permeabilization and apoptosis in the embryo. Apoptosis was induced in *drp1* mutant embryos by X irradiation, which induces expression of Rpr and Hid (Nordstrom et al., 1996; Brodsky et al., 2004). Mitochondria in embryos lacking Drp1 remained intact, as judged by punctate Cyt *c* staining (Figures 7A–7L). In addition, loss of Drp1 decreased the amount of apoptosis as detected by caspase activation (Figure 7M). This correlation between mitochondrial permeabilization and apoptosis induction strongly suggests that mitochondrial permeabilization is an important factor in Rpr-induced and DNA-damage-induced death.

DISCUSSION

A role for mitochondria in apoptosis appears to be conserved from mammals to nematodes to yeast. The lack of clear evidence that mitochondria play a role in *Drosophila* apoptosis has prompted discussion of whether flies

represent an evolutionary outlier in this highly conserved process. Our data strongly suggests that mitochondrial disruption also plays a role in *Drosophila* apoptosis.

Altered Mitochondrial Permeability Is Conserved
Our data show that mitochondria rapidly become permeable to Cyt *c* when Rpr or Hid are expressed, both in cultured cells and in vivo. This alteration in mitochondrial permeability was also seen during DNA-damage-induced apoptosis. Importantly, we demonstrated that the mitochondrial permeabilization during DNA-damage-induced apoptosis was dependent on the genes in the H99 interval. Taken together, these data indicate that Rpr and Hid are both necessary and sufficient for mitochondrial permeabilization.

In contrast, apoptosis induced by Actinomycin D, UV, and DIAP1 RNAi does not result in mitochondrial permeabilization. This indicates that caspase activation alone is not sufficient to induce mitochondrial permeabilization and that the mitochondrial permeabilization we see on Rpr or Hid induction is not simply a general late event in apoptosis. The efficient cell killing by Actinomycin D, UV, and DIAP1 RNAi also implies that mitochondrial permeabilization is not important for all apoptosis in *Drosophila* cells. Rather, it suggests that the Rpr and Hid proteins have a specific activity on the mitochondria that results in mitochondrial permeabilization to execute apoptosis in a timely manner.

The effects of Rpr and Hid on mitochondria were not limited to permeabilization. We found that mitochondrial morphology was dramatically altered within 90 min of Rpr or Hid expression, in both S2 cells and embryos. We found a variety of defects in mitochondrial ultrastructure ranging from a rounded appearance, to bulging (and occasional rupture) of the outer mitochondrial membrane, to swelling of the matrix and disruption of the cristae. This was rarely seen with other inducers of apoptosis. Rpr and Hid may directly cause altered mitochondrial morphology or could act indirectly through other proteins localized at the mitochondria.

The absence of mitochondrial permeabilization in cells treated with DIAP1 dsRNA indicates that the mitochondrial function of Rpr and Hid is independent of their ability to inhibit DIAP1. This is confirmed by our data showing that expression of ΔN-Rpr results in mitochondrial permeabilization despite the fact that this protein lacks the necessary motif to inhibit DIAP1 antiapoptotic activity (Chen et al., 2004). Taken together, these data demonstrate that Rpr and Hid have dual activities in the cell, both to inhibit DIAP1 and to permeabilize mitochondria. Data from other labs have suggested that Rpr is a multifunctional protein (Thomenius and Kornbluth, 2006). Our data confirm that Rpr has multiple proapoptotic activities in the fly.

The dual functionality of Rpr and Hid parallel the recently described role of *C. elegans* Egl-1 in mitochondrial damage (Jagasia et al., 2005). Egl-1 induces apoptosis by binding to Ced-9 to promote both the activation of the caspase Ced-3 and mitochondrial fragmentation. Similarly, Rpr and Hid bind to DIAP1, displacing active caspases and act on mitochondria to promote mitochondrial
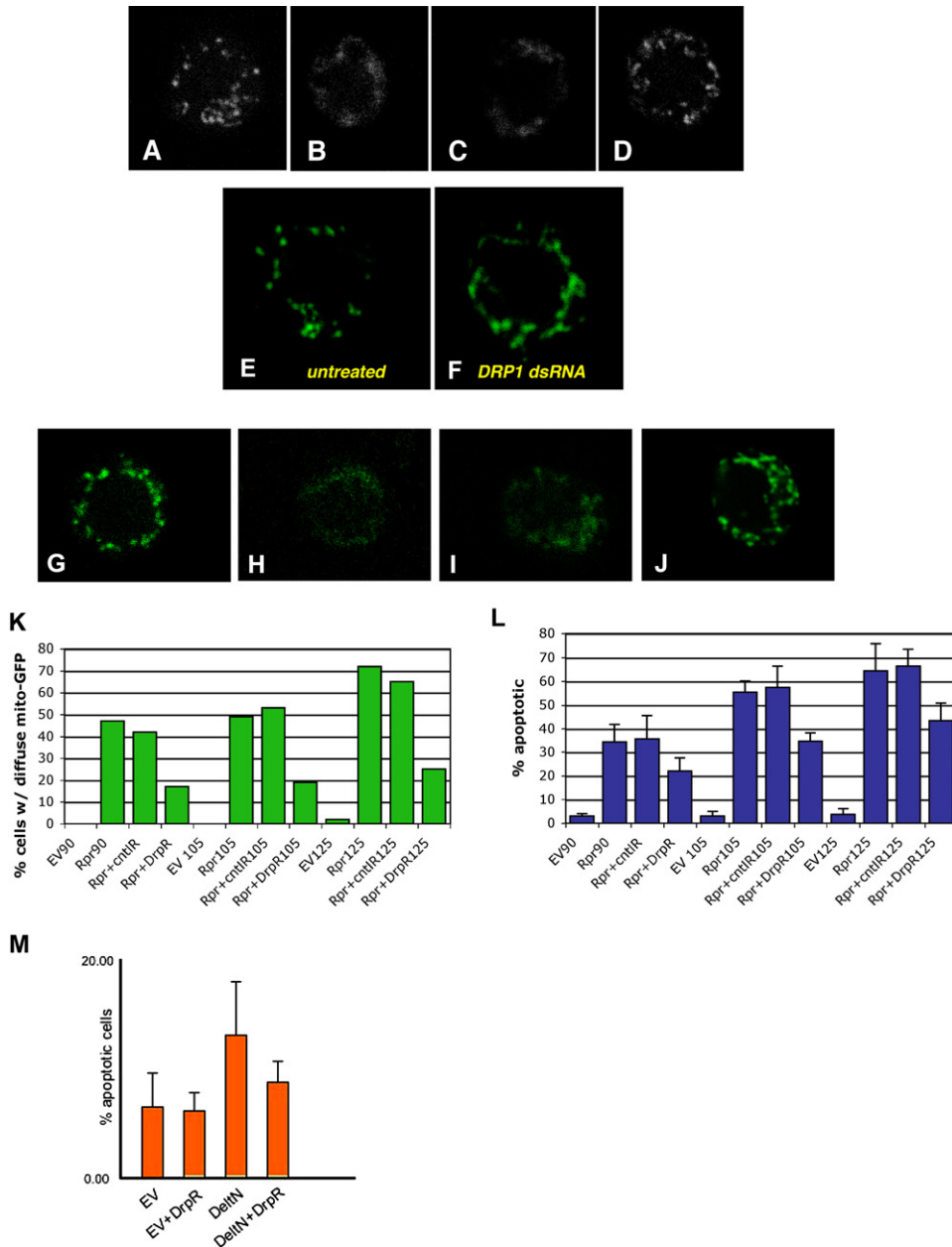
Figure 6. Knockdown of Drp-1 Inhibits Mitochondrial Disruption in Apoptotic Cells and Suppresses Apoptosis

S2 cells were treated with control double-stranded RNA (dsRNA) or dsRNA for Drp-1 and transfected with pMT-Rpr or empty vector. (A–D) After 90 min of Rpr expression, cells were assayed for Cyt *c* localization: (A) empty vector; (B) pMT-Rpr; (C) pMT-Rpr + control dsRNA; (D) pMT-Rpr + Drp-1 dsRNA. Note that loss of Drp-1 blocks permeabilization of the mitochondria to Cyt *c* after Rpr expression. (E and F) Drp1 RNAi alters mitochondrial morphology, as assessed by mito-GFP. Note that the mitochondria are larger and more reticular in the RNAi-treated cells. (G–J) Cells transfected with mito-GFP (LaJeunesse et al., 2004) at 105 min after induction: (G) empty vector; (H) pMT-Rpr; (I) pMT-Rpr + control dsRNA; (J) pMT-Rpr + Drp-1 dsRNA. Knockdown of Drp-1 inhibits loss of mito-GFP. (K) Quantification of data shown in the panels above. The number of apoptotic cells with diffuse or weak mito-GFP was counted at 90, 105, and 125 min after Rpr induction. Note that Drp-1 is required for a significant fraction of the mito-GFP decrease. (L) Loss of Drp-1 inhibits apoptosis, as assessed by cell morphology. The percent of the total cells that appeared apoptotic was counted at 90, 105, and 125 min after Rpr induction (mean ± SD). Drp-1 knockdown (DrpR) significantly decreased the apoptosis induced by Rpr ($p < 0.05$ at 105 and 125 min). (M) Drp-1 knockdown inhibits killing by ΔN-Rpr. Apoptotic cells were scored by morphology at 24 hr after induction (mean ± SD). Empty vector was compared to ΔN-Rpr, with or without Drp-1 ds-RNA.

disruction. One difference between *C. elegans* and flies appears to be the requirement for caspase activity in the mitochondrial disruption. In *C. elegans*, Ced-3 is not required for fragmentation but is required for apoptosis in response to fragmentation. In *Drosophila*, caspase activity participates in the mitochondrial changes.
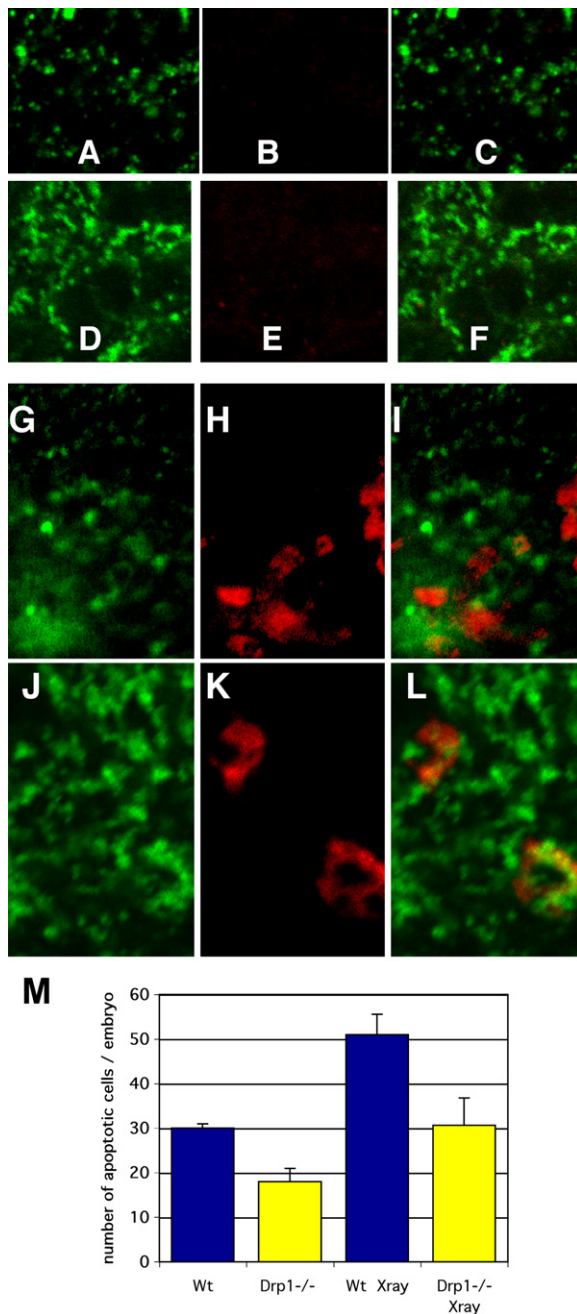
Figure 7. Mutation of Drp1 Inhibits Mitochondrial Permeabilization and Apoptosis in Response to Ionizing Irradiation

Anti-Cyt *c* staining (green) and antiactive caspase 3 staining (red) of unirradiated (A–F) and irradiated (G–L) embryos. Wild-type embryos show normal punctate mitochondria in living cells (A–C) and diffuse Cyt *c* in dying cells after irradiation (G–I). Homozygous Drp1 ^{KG03185} mutant embryos have a more reticular Cyt *c* distribution in living cells (D–F), and Cyt *c* remains more punctate in dying cells (J–L). Also note the altered distribution of active caspase, similar to what is seen in dying cells in the H99 mutants (Figure 2F). There is a decrease in caspase-positive cells in unirradiated and irradiated Drp1 mutant embryos (M) (mean ± SD).

Mitochondrial Events Contribute to *Drosophila* Apoptosis

Two lines of evidence support a role for mitochondrial disruption in *Drosophila* apoptosis. First, Rpr and Hid must localize to mitochondria to elicit a full apoptotic response. Second, if we block mitochondrial disruption by inhibiting Drp1 expression, we see a decrease in apoptosis. Our data clearly indicate that mitochondrial localization of Rpr and Hid is required for a full apoptotic response in S2 cells. This agrees with previous data on Rpr and also with studies on a Grim mutant lacking a mitochondrial localization signal (Claveria et al., 2002; Olson et al., 2003; Chen et al., 2004). Mitochondrial localization of Hid was previously demonstrated in a heterologous system (Haining et al., 1999). In this previous study, Hid killing was not compromised in the absence of mitochondrial localization, in contrast to our observations in *Drosophila* cells. A role for mitochondrial localization is also supported by the finding that two mutant forms of Hid that lack mitochondrial localization in mammalian cells behave as weak loss-of-function alleles in the fly (Grether et al., 1995; Haining et al., 1999).

The mitochondrial fission protein Drp1 is implicated in mitochondrial disruption during apoptosis in yeast, nematodes, and mammals (Frank et al., 2001; Fannjiang et al., 2004; Jagasia et al., 2005). Our data indicate a role for this protein in Rpr-induced and DNA-damage-induced mitochondrial disruption in S2 cells and in the embryo. Furthermore, the inhibition of mitochondrial disruption after Drp1 knockdown is correlated with a decrease in apoptosis, strongly suggesting that mitochondrial disruption contributes to the apoptotic response. It is interesting to note that Drp1 plays a conserved role in apoptosis in a wide variety of organisms but seems to function downstream of different pathways. In mammals, inhibition of Drp1 blocks apoptosis in response to activation of proapoptotic Bcl-2 family members (Frank et al., 2001). In *C. elegans*, Drp1 inhibition blocks endogenous death downstream of Egl1 and Ced9, also Bcl-2 family proteins (Jagasia et al., 2005). Even in yeast, the role of Drp1 in cell death can be modulated by Bcl-2 family proteins (Fannjiang et al., 2004). Surprisingly, in flies, Drp1 appears to be acting downstream of a different family of apoptosis inducers, the RHG proteins. It remains to be seen whether a role for the fly Bcl-2 family proteins can be established in mitochondrial disruption.

How Do Mitochondrial Events Contribute to Apoptosis in the Fly?

Release of apoptogenic factors, most notably Cyt *c*, from the mitochondria is an essential step in most apoptosis in mammalian systems (Kroemer and Reed, 2000). However, our work confirms the findings of others that Cyt *c*, although released from mitochondria by Rpr and Hid, is not important for Rpr or Hid killing (our data and Zimmermann et al., 2002; Dorstyn et al., 2004; Means et al., 2006). It should be noted that Cyt *c* has been shown to be important in some *Drosophila* developmental apoptosis (Arama et al., 2003, 2006; Huh et al., 2004; Mendes et al., 2006). In

these deaths, Hid is likely to act upstream of Cyt *c* release. If Cyt *c* release is required in some cells for Hid-mediated caspase activation, why not in S2 cells? It is possible that there are both Cyt *c*-dependent and -independent mechanisms for activating caspases, and these may be cell-type dependent. Recent data from mice carrying a nonapoptogenic form of Cyt *c* supports this model, as this study suggests that there is both Cyt *c*-dependent and -independent apoptosis during mouse development (Hao et al., 2005).

If release of Cyt *c* is not an essential step in apoptosis in most fly cells, is another apoptosis-inducing factor released during mitochondrial disruption? In mammalian cells, release of other mitochondrial proteins such as SMAC/Diablo, Omi/HTRA2, and AIF are proposed to contribute to apoptosis (Danial and Korsmeyer, 2004). There is some evidence that released mitochondrial factors do not contribute to caspase activation in the fly. Unlike in the mammalian system, mitochondrial lysates cannot activate caspases in fly cytoplasmic lysates (Means et al., 2006). An alternative possibility is that mitochondrial disruption per se might contribute to apoptosis in the fly through inhibition of normal mitochondrial functions essential for cell viability. This might serve as a backup system, to maximize apoptosis in cells that express low levels of the RHG proteins. A similar role for mitochondrial disruption has been proposed in *C. elegans* (Rolland and Conradt, 2006).

In sum, we conclude from these studies that *Drosophila* is not an outlier in evolution with regard to the involvement of mitochondria in the apoptotic process. Rather, our data indicate that mitochondrial changes contribute to *Drosophila* apoptosis. Our findings suggest that we may need to broaden our view of the role of mitochondria in cell death beyond the release of proapoptotic factors to include the general disruption of mitochondria, which ensures that doomed cells have no chance of recovery. Such a model would fit not only the changes seen in mammalian mitochondria, but also those found in yeast, *C. elegans*, and flies as well.

EXPERIMENTAL PROCEDURES

Expression Constructs

Expression plasmids for wild-type and mutant forms of Rpr and Hid have been previously described (Yokokura et al., 2004). The N-terminal Rpr deletion (Δ2–15) mutant (pMT-RprΔN) was inserted into the pRmHa-3 vector. The RprGH3 mutant (L35Q A36R) (Olson et al., 2003) and HidΔC20 mutant (Haining et al., 1999) were constructed by site-directed mutagenesis with GeneTailor (Invitrogen). Similar results were obtained with tagged and untagged constructs.

Cell Culture, Transfection, and Apoptosis Induction

S2 cell cultures were maintained at 25°C and grown in Schneider's *Drosophila* medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen). Cells were transfected according to Yokokura et al. (2004) with 3 μg of pMT-Rpr or 3.75 μg of pMT-Hid with 0.5 μg of pIE-GFP or 2 μg of mito-GFP. The transfection efficiency of these vectors was about 50%. Expression was induced with 700 nmol of $CuSO_4$ for 2×10^6 cells with or without 50 μM (final) zVAD FMK (Calbiochem). Ten μM (final) MG-132 (Calbiochem) was added to samples processed

for western analysis. To obtain equivalent expression of wild-type Rpr and RprGH3 or wild-type Hid and HidΔC20, the expression of the wild-type proteins was induced with 175 nmol or 350 nmol of $CuSO_4$, respectively. Apoptosis was also induced with Actinomycin D (200 nM) for 3, 6, and 8 hr or with UV light (200 mJ/cm²). To induce apoptosis by UV, cells were exposed to UV light in PBS, followed by readdition of media and incubation for 4, 8, 12, and 16 hr. Total cell lysates were assayed for caspase activity as previously described (Yokokura et al., 2004). Percent apoptosis was assessed by morphology.

RNAi Treatment

For Drp1 analysis, cells were transfected with empty vector, pMT-Rpr, or pMT-ΔN-Rpr and 2 μg of Drp-1 dsRNA or Cyt *c*-d control dsRNA. Expression was induced after 36–48 hr. Mitochondrial morphology was visibly altered 36 hr after transfection with Drp-1 dsRNA (Figures 6E and 6F). Expression of Rpr was induced for 90, 105, and 125 min. Expression of ΔN-Rpr was induced for 24 hr. DIAP1 was knocked down by RNAi as described elsewhere (Yokokura et al., 2004). Efficiency of DIAP1 elimination was assessed by caspase activation.

Western Blots

Western blots were performed as described (Yokokura et al., 2004). Ten micrograms of total protein was loaded per lane and detected with anti-HA (1:2000, Covance/Babco clone 16B12), anti-Hid (1:7500), or anti-mouse Cyt *c* (1:500, Zymed, clone 7H8.2C12).

Mitochondrial Permeability Assays

Permeability of mitochondria to Cyt *c* was scored by immunohistochemistry and by FACS analysis (Waterhouse and Trapani, 2003). Briefly, cells (1×10^5) were harvested and permeabilized with digitonin (Fluka Biochemica 50 μg/ml in PBS with 10 mM KCl) for 5 min on ice. Permeabilization of the plasma membrane was verified by Trypan blue. The cells were then analyzed for the release of mitochondrial Cyt *c* by immunohistochemistry as described below and analyzed by the Cutaneous Biology Research Center cell sorter core, by using BD FACSDiVa software (BD Biosciences). In the absence of digitonin, no lower peak was detected after any treatment (data not shown).

Rpr and Hid Expression and X Irradiation of Embryos

Flies were maintained at 25°C. hs-Rpr (White et al., 1996), hs-Hid (Grether et al., 1995), and wild-type embryos were heat shocked at 39°C at 2–4 hr old and kept at room temperature for 90 min before analysis by immunohistochemistry or electron microscopy. To induce apoptosis by X irradiation, 3–4 hr old wild-type and H99 homozygous embryos were X irradiated with 4000 Rads, kept for 5 or 10 hr, and then analyzed by immunohistochemistry as described (White et al., 1994). Drp-1^{KG03185} mutant embryos were collected from homozygous mothers and irradiated as above. Cells positive for anti-active Caspase 3 were counted 10 hr after irradiation in nine embryos of each genotype.

Immunofluorescence and Confocal Microscopy

Immunofluorescence and direct fluorescence microscopy was performed as described (Lisi et al., 2000). S2 cells were plated in a chamber slide (Nalge Nunc International), allowed to attach, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and permeabilized with a buffer containing 0.5% Triton X-100 in PBS. Washes and incubation with antibody were done in 0.1% Tween in PBS for cells. Embryos were dechorionated, fixed, and incubated with primary antibodies including mouse anti-Cyt *c* (Zymed, clone 7H8.2C12 1:200), rabbit anti-HA (Santa Cruz 1:200), and rabbit anti-active caspase 3 (Cell Signaling, 1:200). Secondary antibodies were Alexa 488-conjugated donkey anti-mouse and Alexa 647-conjugated goat anti-rabbit IgG (Molecular Probes 1:200). Nomarski optics was used to assess the morphology of cells undergoing apoptosis. Cells were scored for Cyt *c* distribution in a blinded manner. Mitochondrial localization of anti-Cyt *c* staining was confirmed by labeling S2 cells with MitoTracker Red (Molecular Probes) prior to fixation. For live-cell

imaging, cells were transfected with SpSqEYFP-mito (mito-GFP) (LaJeunesse et al., 2004) with or without pMT-Rpr and with or without Drp1 dsRNA or control dsRNA. Cells were scored in a blinded manner for mito-GFP expression and localization and apoptosis.

Transmission Electron Microscopy

S2 cells were fixed in 2.5% glutaraldehyde/2.5% formaldehyde/0.1 M sodium cacodylate buffer (pH 7.2) for 1.5–2.5 hr at +4°C, washed with 0.1 M sodium cacodylate buffer, and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hr. After washing twice in 0.1 M sodium cacodylate buffer (pH 7.2), specimens were dehydrated in a graded ethanol-water series, infiltrated in 1:1 Spurrs resin for 2 hr to overnight, and immersed for 6 hr to overnight in 100% resin. Blocks were placed in fresh Spurrs resin in Beem capsules, polymerized at 55°C –60°C for 24 hr, and allowed to cure for 24 hr before sectioning. Thin sections (silver/gold) were stained with uranyl acetate and lead citrate and observed on a JEOL JEM 1010 transmission electron microscope. Experiments were repeated at least three times. Embryos were heat shocked as described above and fixed in 3% glutaraldehyde/1.5% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Sciences) for 1.5–2.5 hr at +4°C. They were then processed for EM by the above protocol. Twenty to fifty cells for each treatment were scored for the presence of two or more abnormal mitochondria in a cell.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Two Figures, and One Table and are available at http://www.developmentalcell.com/cgi/content/full/12/5/793/DC1/.

REFERENCES

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

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

Bangs, P., and White, K. (2000). Regulation and execution of apoptosis during *Drosophila* development. Dev. Dyn. *218*, 68–79.

Brodsky, M.H., Weinert, B.T., Tsang, G., Rong, Y.S., McGinnis, N.M., Golic, K.G., Rio, D.C., and Rubin, G.M. (2004). *Drosophila melanogaster* MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. Mol. Cell. Biol. *24*, 1219–1231.

Chen, F., Hersh, B.M., Conradt, B., Zhou, Z., Riemer, D., Gruenbaum, Y., and Horvitz, H.R. (2000). Translocation of *C. elegans* CED-4 to nuclear membranes during programmed cell death. Science *287*, 1485–1489.

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

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

Conradt, B., and Horvitz, H.R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. Cell *93*, 519–529.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205–219.

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

Dorstyn, L., Mills, K., Lazebnik, Y., and Kumar, S. (2004). The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in *Drosophila* cells. J. Cell Biol. *167*, 405–410.

Fannjiang, Y., Cheng, W.C., Lee, S.J., Qi, B., Pevsner, J., McCaffery, J.M., Hill, R.B., Basanez, G., and Hardwick, J.M. (2004). Mitochondrial fission proteins regulate programmed cell death in yeast. Genes Dev. *18*, 2785–2797.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell *1*, 515–525.

Goyal, L. (2001). Cell death inhibition: keeping caspases in check. Cell *104*, 805–808.

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

Haining, W., Carboy-Newcomb, C., Wei, C., and Steller, H. (1999). The proapoptotic function of *Drosophila* Hid is conserved in mammalian cells. Proc. Natl. Acad. Sci. USA *96*, 4936–4941.

Hao, Z., Duncan, G.S., Chang, C.C., Elia, A., Fang, M., Wakeham, A., Okada, H., Calzascia, T., Jang, Y., You-Ten, A., et al. (2005). Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. Cell *121*, 579–591.

Huh, J.R., Vernooy, S.Y., Yu, H., Yan, N., Shi, Y., Guo, M., and Hay, B.A. (2004). Multiple apoptotic caspase cascades are required in nonapoptotic roles for *Drosophila* spermatid individualization. PLoS Biol. *2*, E15.

Igaki, T., Suzuki, Y., Tokushige, N., Aonuma, H., Takahashi, R., and Miura, M. (2007). Evolution of mitochondrial cell death pathway: proapoptotic role of HtrA2/Omi in *Drosophila*. Biochem. Biophys. Res. Commun. *356*, 993–997.

Jagasia, R., Grote, P., Westermann, B., and Conradt, B. (2005). DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in *C. elegans*. Nature *433*, 754–760.

Karbowski, M., Lee, Y.J., Gaume, B., Jeong, S.Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C.L., and Youle, R.J. (2002). Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. J. Cell Biol. *159*, 931–938.

Kornbluth, S., and White, K. (2005). Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). J. Cell Sci. *118*, 1779–1787.

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

LaJeunesse, D.R., Buckner, S.M., Lake, J., Na, C., Pirt, A., and Fromson, K. (2004). Three new *Drosophila* markers of intracellular membranes. Biotechniques *36*, 784–788, 790.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S., Ahmad, M., Alnemri, E., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell *91*, 479–489.

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

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

Mendes, C.S., Arama, E., Brown, S., Scherr, H., Srivastava, M., Bergmann, A., Steller, H., and Mollereau, B. (2006). Cytochrome c-d regulates developmental apoptosis in the *Drosophila* retina. EMBO Rep. *7*, 933–939.

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

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

Rizzuto, R., Brini, M., Pizzo, P., Murgia, M., and Pozzan, T. (1995). Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells. Curr. Biol. *5*, 635–642.

Rolland, S., and Conradt, B. (2006). The role of mitochondria in apoptosis induction in *Caenorhabditis elegans*: more than just innocent bystanders? Cell Death Differ. *13*, 1281–1286.

Smiley, S.T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T.W., Steele, G.D., Jr., and Chen, L.B. (1991). Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc. Natl. Acad. Sci. USA *88*, 3671–3675.

Thomenius, M., and Kornbluth, S. (2006). Multifunctional reaper: sixty-five amino acids of fury. Cell Death Differ. *13*, 1305–1309.

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

Verstreken, P., Ly, C.V., Venken, K.J., Koh, T.W., Zhou, Y., and Bellen, H.J. (2005). Synaptic mitochondria are critical for mobilization of reserve pool vesicles at *Drosophila* neuromuscular junctions. Neuron *47*, 365–378.

Waterhouse, N.J., and Trapani, J.A. (2003). A new quantitative assay for cytochrome c release in apoptotic cells. Cell Death Differ. *10*, 853–855.

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

White, K., Tahaoglu, E., and Steller, H. (1996). Cell killing by the *Drosophila* gene *reaper*. Science *271*, 805–807.

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

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

Yu, X., Wang, L., Acehan, D., Wang, X., and Akey, C.W. (2006). Three-dimensional structure of a double apoptosome formed by the *Drosophila* Apaf-1 related killer. J. Mol. Biol. *355*, 577–589.

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