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Distinct Pathways Mediate UV-Induced Apoptosis in *Drosophila* Embryos

Lei Zhou[1] and Hermann Steller*[,2]
Howard Hughes Medical Institute
Department of Biology
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Summary

Cell death in *Drosophila* is regulated by many of the same signals that control apoptosis in mammalian systems. For all the three major cell death pathways that have been described in humans, homologous components have been identified in *Drosophila*. Here we report that distinct pathways mediate UV-induced apoptosis at different developmental stages in the *Drosophila* embryo. In midstage embryos, UVC irradiation induces *reaper* expression and cell death through a *mei-41*(dATM)-dependent pathway; UVB does not have the same effect. In contrast, in pregastrulation embryos, both UVB and UVC promote apoptosis via transcriptional induction of the *Drosophila* Apaf-1/*ced-4* homolog. This early UV response requires E2F but not *mei-41* function and appears to be independent of DNA damage.

Introduction

In multicellular organisms, cells that have been irreversibly damaged by environmental stress typically undergo apoptosis, a morphologically distinct form of programmed cell death (Hengartner, 2000). This active removal of damaged cells limits the accumulation of harmful cells that might otherwise endanger the survival of the organism. Inducing cell death through irradiation and/or cytotoxic drugs is also the major clinical approach to eliminate cancer cells. However, the sensitivity to irradiation or drug treatment can vary dramatically among different cell types and among different types of cancer. The molecular basis for these differences and the exact pathways by which cytotoxic agents induce apoptosis are only incompletely understood. Checkpoint proteins such as P53 have been shown to play important roles in mediating radiation-induced apoptosis, both in human and in insects (Steller, 2000). However, the status of p53 is not always correlated with sensitivity to irradiation-induced cell killing (Zhivotovsky et al., 1999; Zhou et al., 2003). Therefore, radiation-induced apoptosis appears to be controlled by multiple pathways that appear to be differentially utilized in different cellular contexts.

Apoptosis induced by radiation and cytotoxic agents involves caspase activation (Kaufmann and Earnshaw, 2000). To date, three major regulatory pathways for the regulation of caspase-based death programs have been identified: the "mitochondrial pathway," which involves Bcl-2 family proteins and *ced-4*/Apaf-1 (Horvitz, 1999), the *reaper*-family/inhibitor of apoptosis protein (IAP) pathway (Martin, 2002), and the death receptor pathway (Nagata, 1999). Components of all three pathways have been described in both mammals and insects, and perturbations of each of these pathways have been implicated in a variety of human diseases.

The *reaper*/IAP pathway was first characterized in insects (Abrams, 1999). IAPs can directly bind to and inhibit caspases, and Reaper-like proteins, including mammalian Smac/Diablo and Omi/Htra2, can relieve this inhibition. In *Drosophila*, *diap1* (*Drosophila* inhibitor of apoptosis protein-1) is universally expressed and is essential to prevent the inappropriate activation of caspases (Goyal, 2001). The induction of apoptosis in this organism generally requires the activity of three closely linked genes, *reaper*, *grim*, and *head involution defective* (*hid*) (Abrams, 1999). *Reaper*, *grim*, and the recently identified *sickle* gene are tanscriptionally activated in response to many proapoptotic signals, including radiation (Nordstrom et al., 1996; Christich et al., 2002). Besides releasing caspases from Diap1 binding, Reaper and Grim stimulate the intrinsic E3 ubiquitin ligase activity to promote self-ubiquitination and proteosome-mediated degradation of Diap1 protein (Martin, 2002).

Components of the mitochondrial and the death receptor pathways have also been identified in *Drosophila* (Richardson and Kumar, 2002). The *Drosophila* homolog of Apaf-1, *dapaf-1*/*dark*/*hac-1*, induces caspase activation in the presence of ATP and cytochrome C in vitro (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). Genetic interaction studies suggest that the activities of *dapaf-1*/*dark*/*hac-1* and Diap1 converge at the same caspases, suggesting a "gas (Apaf-1) and brake (IAP)" model of caspase activation (Zhou et al., 1999). In mammals, a similar convergence is seen for the regulation of caspase-9 (Du et al., 2000; Srinivasula et al., 2001).

The existence of multiple converging regulatory pathways for caspase activation raises many important questions about the role of any specific pathway in a distinct apoptotic paradigm and how the activities of different pathways are coordinated. In this study, we investigated the function of the *Drosophila* Apaf-1 homolog and the Reaper/Grim/Hid/Diap1 pathway during radiation-induced apoptosis in the *Drosophila* embryo. Surprisingly, we find that different pathways mediate UV radiation-induced cell death at different developmental stages.

Results

UV Irradiation Can Induce *hac-1* Expression and Apoptosis in Pregastrulation *Drosophila* Embryos

During *Drosophila* embryonic development, there is normally no detectable programmed cell death prior to

*Correspondence: steller@techunix.technion.ac.il
[1]Present address: Department of Molecular Genetics and Microbiology, UF Shands Cancer Center, College of Medicine, UFL, Gainesville, Florida 32610.
[2]Present address: Howard Hughes Medical Institute, Strang Laboratory of Cancer Biology, The Rockefeller University, New York, New York 10021.

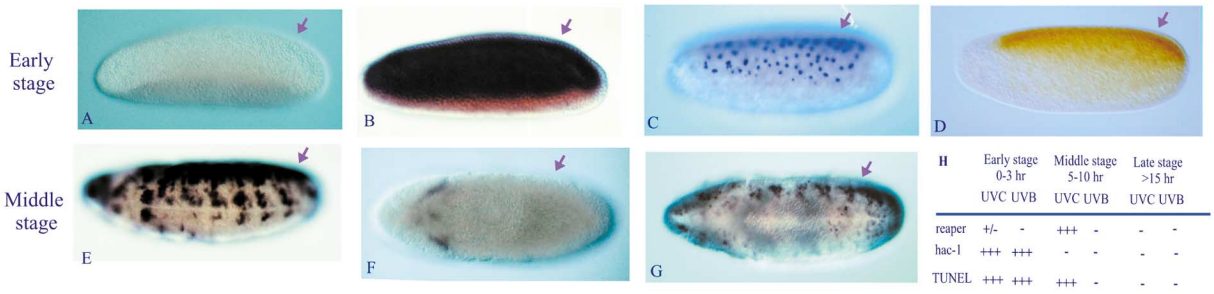

Figure 1. UV-Induced Gene Expression and Apoptosis Depends on the Developmental Stage

When early stage (0–3 hr AEL) embryos were irradiated with UVB (or UVC), *hac-1* but not *reaper* was induced on the irradiated side ([A and B], ISH for *reaper* and *hac-1* in stage 5 embryos, respectively, D-anti-β-Gal labeling of *lacZ* expression in UV-irradiated embryos carrying P{lacZ} in the *hac-1* locus). In contrast, in middle stage (5–8 AEL) embryos, *reaper* but not *hac-1* (E and F, respectively) was induced by UVC. In early stages, both UVC and UVB induced TUNEL-positive cell (nuclei) on the exposed side (C), but only UVC could induce cell death in the mid-stage embryos (G). (H) illustrates how gene expression and the apoptotic response to UV change during the course of *Drosophila* embryogenesis.

stage 10/11, corresponding to about 7 hr after fertilization (at 25°C) (Abrams et al., 1993). However, apoptosis can be induced 4–5 hr after fertilization by ionizing radiation or upon inactivation of *diap1* (Abrams et al., 1993; Wang et al., 1999; Goyal et al., 2000).

During the first 130 min of *Drosophila* embryonic development, the fertilized nucleus undergoes 13 rapid mitotic cycles within a syncitium (Campos-Ortega and Hartenstein, 1985). Cell membranes are formed only after approximately 3 hr. At this stage, termed cellular blastoderm, cells are still omnipotent (Campos-Ortega and Hartenstein, 1985). The apparent absence of apoptosis during the first 4 hr of embryonic development raises the possibility that early embryos are resistant to the induction of apoptosis.

Since early *Drosophila* embryos are highly sensitive to UV irradiation (Nothiger and Strub, 1972), we investigated the effects of UV on apoptosis, *reaper* and *hac-1 (dapaf-1/dark)* expression. To our surprise, we found that UVC induced *hac-1* expression in embryos prior to the formation of cellular blastoderm (Figures 1B and 1D). In contrast, no expression of *reaper* could be detected on the exposed side at this stage. The significance of the induction of *hac-1* expression on the UVC-irradiated side of the embryo was confirmed using three different approaches. First, the increase of *hac-1* mRNA was detected via in situ hybridization. Second, we also saw an increase of Hac-1 protein using a polyclonal antibody raised against a peptide correspond to the N-terminal 14 amino acids (data not shown). Third, we assessed the expression of the *lacZ* gene, using an anti-β-Gal monoclonal antibody, in embryos that carry an enhancer trap P insertion in the promoter region of *hac-1* (Figure 1D). All of the three approaches indicated that *hac-1* is specifically induced on the irradiated side of the embryo. The shielded side of the embryo did not have elevated level of *hac-1* expression. Since the ooplasm is quite opaque to UV light (Togashi and Okada 1983), very little UV light is expected to reach the shaded side.

Coincident with *hac-1* induction, we observed the induction of apoptotic markers upon UV irradiation. In blastoderm embryos, *hac-1* induction by UV was detectable as early as 15 min after irradiation. Large amounts of TUNEL-positive cells appeared at the irradiated side within 40 min after irradiation. Significantly, even prior to cell formation, nuclei on the exposed side became TUNEL positive in UV-irradiated embryos, while nuclei on the shielded side remain TUNEL negative (Figure 1C). This demonstrates that the basic cell death program is present in the early *Drosophila* embryo before actual cells form.

We also found that *hac-1* expression and apoptosis could be induced by UVB but not UVA. The efficacy of UVB for inducing *hac-1* and apoptosis in early stage embryos was in the same range as that of UVC. When embryos were dechorionated, 5 mJ/cm^2 UV-B- or UV-C-induced *hac-1* expression and TUNEL-positive nuclei in 50%–70% of irradiated embryos. To minimize the impact of dechorionation as an additional stress, most of our experiments were done using embryos with an intact chorion irradiated at 50 mJ/cm^2 UV-B or UV-C, unless otherwise stated. It has been reported that the egg shell blocks about 80% of UV energy (Kalthoff, 1971).

The UV Response Changes during Embryogenesis

To examine the effect of embryonic age on UV-induced apoptosis, we irradiated embryos at different developmental stages with UVC or UVB and examined the expression of *reaper*, *hac-1*, and TUNEL (Figure 1H). In early embryonic stages, prior to the onset of gastrulation (0–3 hr after fertilization), both UVC and UVB irradiation induced *hac-1* expression and TUNEL, but not *reaper* (Figures 1A–1C). However, slightly older embryos (5–10 hr) displayed a strikingly different response (Figures 1E–1G). By stage 9–10, the response of *hac-1* was completely lost, but UVC was still able to induce TUNEL. Interestingly, as the *hac-1* response was attenuated, *reaper* transcription became inducible by UVC, but not UVB irradiation. In late embryos (12 hr after fertilization and later), neither *hac-1* nor *reaper* could be induced by the same dosage of UVC (or UVB), and these embryos were also resistant to the induction of apoptosis under these conditions (Figure 2H). Furthermore, even much higher UV doses (500 mJ/cm^2) failed to induce detectable level of TUNEL-positive cells and *reaper/hac-1* expression at this stage (data not shown). Therefore, the

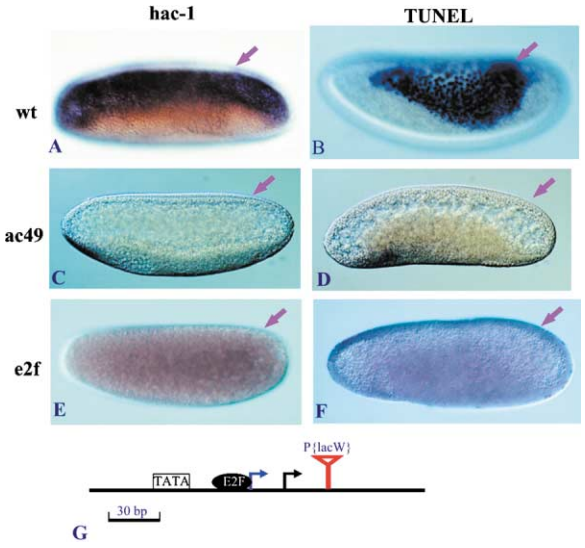

Figure 2. Induction of *hac-1* Expression Is Required for UV-Induced Nuclear DNA Degradation

UV induced *hac-1* expression 15 min after UV irradiation in a wild-type embryo (A). This induction was absent in homozygous *hac-1[ac49]* mutant embryos (C) as well as embryos from trans-heterozygous e2f mutants (E). TUNEL labeling of apoptotic nuclei was performed on embryos fixed 45 min after irradiation. In wild-type embryos, TUNEL-positive nuclei are involuted into the embryo (B). No TUNEL-positive nuclei were detected in *hac-1[ac49]* mutant embryos and involution was not observed (D). TUNEL labeling on the irradiated side was also absent in embryos lacking *e2f* (F). Arrows indicate the side of the embryo exposed to UV. (G) Relative position of the P insertion and the E2F binding site in the *hac-1* promoter. The blue arrow marks the predicted transcription start site, and the black arrow indicates the 5′ end of the longest cDNA clone (SD15113).

induction of *hac-1* and/or *reaper* corresponded well with the induction of apoptosis by UV in *Drosophila* embryos. This prompted us to address whether the induction of *hac-1/reaper* was required for UV to induce apoptosis.

Elevated *hac-1* Expression Is Required for UV-Induced Apoptosis in Early Embryogenesis

A P element inserted in the promoter region of *hac-1* causes a loss-of-function phenotype (Zhou et al., 1999). Although this allele is in general hypomorphic and viable, it behaves as a complete loss-of-function allele for the embryonic UV response. In embryos homozygous for this allele, *hac-1* was no longer induced by our standard dose (50 mJ/cm^2) of UV irradiation (Figure 2C). Moreover, *hac-1*$^{-/-}$ embryos derived from homozygous mutant parents were completely protected against apoptosis under these conditions TUNEL (Figure 2D). In response to UV, wild-type *Drosophila* embryos underwent a striking morphological response that involved involution of TUNEL-positive nuclei into the embryonic yolk (Figure 2B). Eventually, the irradiated side was covered by cells that apparently migrated in from the shielded side. After involution of the damaged nuclei, TUNEL labeling gradually disappeared, presumably as the consequence of nuclear degradation. In *hac-1*$^{-/-}$ embryos, no involution of nuclei was observed. Rather, an "open wound" remained at the irradiated side (Figure 2D). These observations indicate a strict requirement for *hac-1* during UV-induced apoptosis in early *Drosophila* embryos. Furthermore, it appears that apoptosis of damaged nuclei is necessary for their removal and to "repair" early embryonic damage.

We identified an E2F consensus binding site within the core promoter region of *hac-1*, between the TATA-box and the P element insertion (Figure 2G). Since E2F binding site within core promoter regions are often important for gene expression, we tested whether *e2f* function is required for UV-induced *hac-1* expression. Since *e2f* function is maternally contributed to the *Drosophila* embryo, we sought to generate embryos derived from mutant mothers. Although most *Drosophila* loss-of-function alleles of *e2f* are lethal, some trans-heterozygous combinations are viable and lay eggs (Royzman et al., 1999). Significantly, UV no longer induced *hac-1* expression and apoptosis (as assayed by TUNEL staining) in embryos derived from mutant mothers (Figures 2E and 2F), demonstrating that *e2f* activity is required for early UV-mediated *hac-1* transcription. Interestingly, human E2F is known to regulate mammalian *apaf-1* (Moroni et al., 2001). Because most of the embryos laid by trans-heterozygous *e2f* mutant mothers failed to develop beyond cellular blastoderm, it was not possible to investigate the requirement of E2F for *hac-1* and/or *reaper* transcription at later developmental stages.

mei-41(dATM) Is Required for Irradiation-Induced Expression of Reaper but Not *hac-1*

In mammalian systems, UVC-induced DNA aberrations are detected by a family of DNA associated kinases including ATM, ATR (Zhou and Elledge, 2000). In *Drosophila*, *mei-41* has been identified as the structural and functional homolog of ATM/ATR (Hari et al., 1995). *mei-41* null mutants are viable, but they are highly sensitive to mutagens, such as X-ray and UV irradiation (Boyd et al., 1976; Smith, 1976). To test whether *mei-41* function is required for UV induction of *hac-1* or reaper, we exposed *mei-41* mutant embryos to UVC and UVB irradiation at different developmental stages. Both UVC and UVB induced *hac-1* expression and caused TUNEL-positive nuclei in embryos (data not shown). Therefore, neither zygotic nor maternal *mei-41* function is required for *hac-1* expression. In contrast, the induction of apoptosis and reaper by UVC in midstage embryos was completely blocked in *mei-41* mutant embryos (Figure 3). On the other hand, the normal developmental profile of reaper expression and apoptosis was not affected in *mei-41* mutant embryos. We conclude that *mei-41* mediates a distinct apoptotic pathway in response to UVC that involves reaper but not *hac-1*.

The Primary Target for UV-Induced *hac-1* Expression Appears to Be Cytoplasmic

The fact that UV-induced *hac-1* expression does not require *mei-41* function led us to further investigate whether *hac-1* induction is mediated by nuclear DNA damage. For this purpose, we utilized a developmental feature of the *Drosophila* embryo. At the beginning of embryogenesis, the fusion of the sperm and egg nuclei occurs roughly in the middle of the embryo, and the

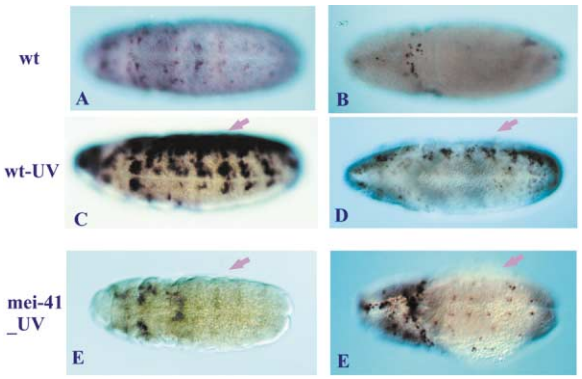

Figure 3. *mei-41* Function Is Required for UV-Induced *reaper* Expression and Apoptosis in Middle Stage Embryos

(A) and (B) are wild-type stage 10/11 embryos without UV treatment processed for *reaper* in situ and TUNEL labeling. Wild-type (C and D) and *mei-41*[D5] mutant (E and F) embryos irradiated with 50 mJ/cm^2 UVC were monitored for *reaper* expression (C and E) and TUNEL (D and F). Exposed side is up for all UV-treated embryos. Coinciding with the absence of UV-induced *reaper* expression (E versus C), there were no UV-induced TUNEL-positive cells on the irradiated side (arrow) of *mei-41* mutant embryos (F versus D).

fused nuclei remain in a central position slightly closer to the anterior end of the embryo (Figure 4A). For the first eight mitotic cycles, corresponding to approximately 1 hr of development, the nuclei stay in the center of the egg. Due to the opacity of the ooplasm, less than 1% of the UV is expected to reach the nuclei during the early cleavage stages. However, when we irradiated eggs within 10 min of egg laying, we observed that *hac-1* was induced on the irradiated side once the nuclei migrated to the periphery, about 1 hr after irradiation (Figure 4B). These nuclei also became TUNEL positive (Figure 4C). These observations suggest that the primary target for UV is cytoplasmic and not direct nuclear DNA damage. It appears that this cytoplasmic "signal" persists until nuclei reach the irradiated zone. Significantly, this ability to "sense" and "transduce" UV irradiation to activate *hac-1* transcription is a special feature of the early *Drosophila* embryo, since this response was lost within 4–5 hr after fertilization.

Discussion

UV-induced ("sunburn") cell damage was observed many decades ago. It is clear now that "sunburn" is the result of keratinocytes undergoing apoptosis (Kulms and Schwarz, 2000). Although UV irradiation has been used as a popular means of inducing cell death in laboratory systems, there remains controversy regarding the underlying molecular pathways. There is consensus that UV-induced apoptosis is a consequence of nuclear DNA damage (pyrimidine dimers). According to this model, the DNA damage is sensed by the ATM family of DNA associating protein kinases, which in turn activate the tumor suppressor protein p53. Once activated, p53 is thought to induce apoptosis via directly activating cell death regulatory genes. Several proapoptotic genes have been identified as potential direct targets of p53 transcriptional regulatory activity, including *bax*, *Fas/CD95*, DR5, *reaper*, *pidd*, and others (reviewed in Vousden, 2000; Zhou et al., 2003). However, UV irradiation can also activate signal transduction mechanisms independent of DNA damage. For example, JNK and NFκB can be activated in enucleated cells upon UV irradiation (Devary et al., 1993).

The involvement of multiple cell death regulatory pathways in UV-induced apoptosis raises several important

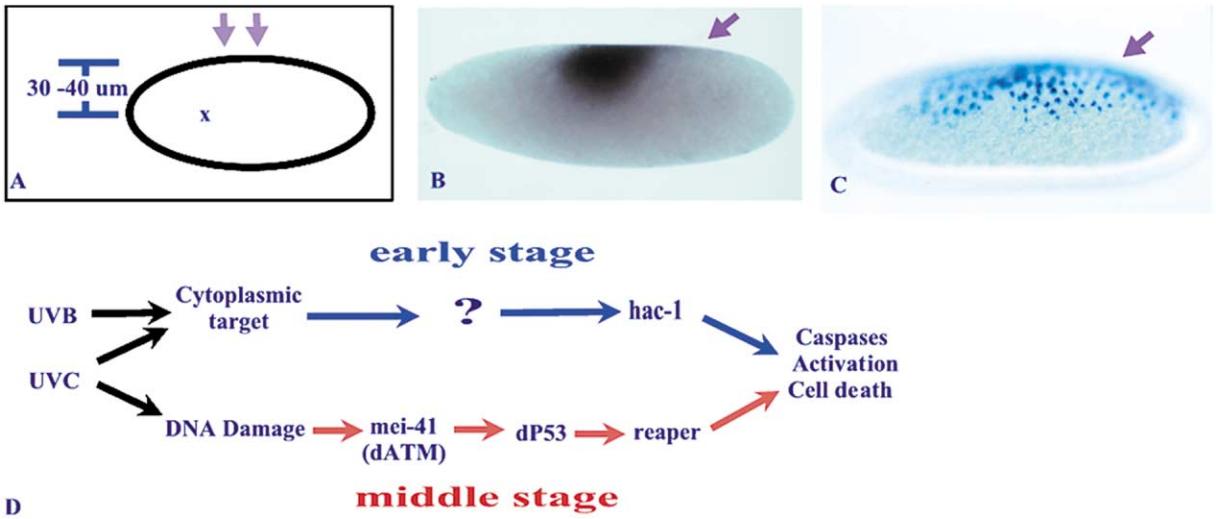

Figure 4. Induction of *hac-1* by UV Is Independent of Nuclear DNA Damage

(A) indicates the site of the nucleus (x) at the time of UV irradiation (arrow). Less than 1% of the original energy was expected to reach the nucleus, as 50% is absorbed for every 3–4 μm ooplasm. When early zygotes within 10 min of egg laying were irradiated with UVB or UVC, *hac-1* was induced as soon as the nuclei moved to the periphery (B), and these nuclei became TUNEL positive (C). (G) Two distinct pathways mediate UV-induced cell death at different development stages.

questions about their relative contribution and possible coordination. In the past, most of the relevant mechanistic studies were performed on specific cell lines grown in tissue culture. In this study, we investigated the UV response of cells in the intact *Drosophila* embryo. Surprisingly, we found that different pathways were used at different developmental stages to mediate UV-induced cell death. Therefore, it appears that cells can rapidly and dramatically change pathways that mediate radiation-induced apoptosis. These results illustrate the problems in generalizing results obtained for one specific cell type and/or differentiation stage.

The *mei-41*-Dependent Pathway

We found that the induction of apoptosis and *reaper* expression by UV depend on *mei-41* (dATM) function in midstage embryos. However, *reaper* is not the only transcriptional target of Mei-41, and other *Drosophila* proapoptotic genes are also induced upon UVC irradiation (L.Z. and G. Chan, unpublished data). The functional contribution of these other genes to radiation-induced apoptosis remains to be established.

The *Drosophila* homolog of P53 (dP53) is required for ionizing irradiation-induced *reaper* expression and apoptosis (Brodsky et al., 2000; Ollmann et al., 2000), and *reaper* is a direct target for dP53 (Brodsky et al., 2000). Furthermore, although it is likely that there are additional dP53 targets, *reaper* is required for a normal response to radiation (Peterson et al., 2002). Given that UVC-induced *reaper* expression is dependent on Mei-41 (dATM) function, it is very likely that similar pathways involving *mei-41*/ATM and p53 operate in both mammals and insects. This *mei-41*-dependent response to UV irradiation was not observed in embryos older than stage 12, when epidermal cells become postmitotic (Foe et al., 1993). This suggests that, as observed in mammalian systems, the sensitivity to UV irradiation may be related to cell proliferation.

The Primary Target for UV in Early Embryos Appears to Be Cytoplasmic

In contrast to midstage embryos, we found that apoptosis induced by UVC and UVB in early (pregastrulation) embryos did not involve the *mei-41*/*reaper* pathway. Rather, it appears that the *Drosophila* Apaf-1 homolog is a key target for the induction of apoptosis. Also, unlike the *mei-41*-dependent pathway, this pathway is responsive to UVB irradiation as well.

It has been previously noticed that UV irradiation of zygotes can induce localized developmental defects. When the posterior region of the early *Drosophila* egg is irradiated, defects in pole cell formation result (Nothiger and Strub, 1972; Togashi and Okada, 1983). Since UV light does not penetrate the ooplasma well, the zygote nucleus is highly protected from DNA damage. In fact, there is direct experimental evidence that UV-induced defects in early insect embryos are independent of nuclear DNA damage, but the underlying mechanism has remained obscure. Our results on *hac-1* induction provide a molecular readout of these early cytoplasmic changes. When embryos were irradiated during the very early cleavage cycles, *hac-1* was specifically induced in the nuclei that migrated to the irradiated side of the embryo. Furthermore, the subsequent apoptosis and elimination of these nuclei dependent on *hac-1* function, demonstrated that the *Drosophila* Apaf-1 homolog is required for this process. In *hac-1* mutant embryos, irradiated embryos retained an "open wound" and eventually degenerated. We conclude that *hac-1*-mediated apoptosis is important to remove damaged cells, thereby providing the embryo with developmental plasticity to respond to and repair damage.

Why Are Multiple Pathways Involved in UV-Induced Cell Death?

On the surface, the use of two independent pathways for the induction of apoptosis in response to the same stimulus appears redundant. However, it seems that the two distinct pathways implicated in this study for UV-induced apoptosis mediate cell death in different cellular contexts. While undifferentiated cells require the *Drosophila* Apaf-1 homolog but not *reaper* to induce apoptosis, the converse is seen for differentiating cells. A similar dependence on the state of cell differentiation has been seen in mammalian cells. For example, UVB-induced apoptosis in murine keratinocytes can be mediated by P53-independent or P53-dependent mechanisms. Although the P53-dependent mechanism is predominant in differentiated cells, UVB-induced cell death in undifferentiated keratinocytes is mediated by a P53-independent mechanism (Tron et al., 1998). More generally, there are many examples of differential susceptibility to the induction of apoptosis. In *Drosophila*, cell killing by *reaper* and other proapoptotic proteins varies drastically between different development stages and/or in different cell types (White et al., 1996; Zhou et al., 1997). Whereas ectopic expression of *reaper*- or *hid*-induced apoptosis efficiently in stage 9–13 embryos, no significant killing was observed in younger embryos (unpublished data). The selective activation and utilization of specific apoptotic pathways is of great interest, but little is known about the mechanisms responsible for these stage- and tissue-specific differences. In this regard, radiation-induced apoptosis offers a relatively simple and well-defined paradigm. The future identification and characterization of the upstream signaling pathway should greatly enhance our understanding of radiation-induced apoptosis.

Experimental Procedures

Fly Strains

Canton-S or yw67C23 strains were used as wild-type. *mei-41*[2], *mei-41*[D5], *mei-41*[D9], *mei-41*[D14], *mei-41*[RT1] were obtained from the Bloomington stock center. e2f[91], e2f[i1] and e2f[i2] strains were kindly provided to us by Dr. Orr-Weaver. The P element insertion line l(2)k11502/CyO (P1041) was obtained from the Bloomington stock center. A background lethal mutation was removed to produce viable homozygous strains. The P insertion site was verified via plasmid rescue.

UV Irradiation

Embryos were collected from wild-type or mutant flies and aged at 25°C for various times. A Stratalinker 2400 (Stratagene, La Jolla, CA) was used for UV irradiation. Three different sets of bulbs were used to produce UVC, UVB, and UVA light sources, respectively. Embryos were washed with distilled water and then spread out on clean egg plates as a monolayer and exposed to 5 mJ/cm^2 to 500 mJ/cm^2 irradiation. The embryos were allowed to recover in dark

for either 15–30 min (to monitor gene expression) or for 40–75 min (to monitor cell death) at 25°C, followed by 4% paraformaldehyde fixation. To test the effect of UV irradiation on zygotes, eggs within 10 min of egg laying were irradiated with 50 mJ/cm^2 UVB.

Histology

In situ hybridization and immunocytochemistry were performed essentially as described before (Zhou et al., 1997). TUNEL labeling was performed as previously described (White et al., 1994).

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