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Methods for Analyzing Checkpoint Responses in *Caenorhabditis elegans*

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Summary

In response to genotoxic insults, cells activate DNA damage checkpoint pathways that stimulate DNA repair, lead to a transient cell cycle arrest, and/or elicit programmed cell death (apoptosis) of affected cells. The *Caenorhabditis elegans* germ line was recently established as a model system to study these processes in a genetically tractable, multicellular organism. The utility of this system was revealed by the finding that upon treatment with genotoxic agents, premeiotic *C. elegans* germ cells transiently halt cell cycle progression, whereas meiotic prophase germ cells in the late pachytene stage readily undergo apoptosis. Further, accumulation of unrepaired meiotic recombination intermediates can also lead to the apoptotic demise of affected pachytene cells. DNA damage-induced cell death requires key components of the evolutionarily conserved apoptosis machinery. Moreover, both cell cycle arrest and pachytene apoptosis responses depend on conserved DNA damage checkpoint proteins. Genetics- and genomics-based approaches that have demonstrated roles for conserved checkpoint proteins have also begun to uncover novel components of these response pathways. In this chapter, we will briefly review the *C. elegans* DNA damage-response field, and we will discuss in detail the methods that are being used to assay DNA damage responses in *C. elegans.*

Key Words: *C. elegans*; *C. elegans* germ line; *C. elegans* methods; apoptosis; programmed cell death; DNA damage responses; *ced* genes; germ line; checkpoint responses; p53; *cep-1;* cell cycle arrest; RNAi; RNAi feeding; co-suppression; meiosis; recombination.

1. Introduction

The correct maintenance and duplication of genetic information is constantly challenged by genotoxic stress. Such stress may result from exogenous insults, such as exposure to ionizing radiation or genotoxic chemicals, or may arise by endogenous means—e.g., mistakes in DNA replication, or oxidative damage as a result of intracellular reactive oxygen species. In response to genotoxic

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stress, cells activate checkpoint pathways that lead to (1) DNA repair; (2) a transient cell cycle arrest in order to allow for time to repair compromising genetic lesions; or (3) apoptosis to trigger the demise of genetically damaged cells that might potentially become harmful to the entire organism.

1.1. Organization of the Nematode Germ Line

To investigate DNA damage-induced apoptosis and cell cycle arrest in a multicellular, genetically tractable model organism, we began to employ the germ line of the nematode worm *Caenohrabditis elegans* as an experimental system ***(1)***. In contrast to classical studies of apoptosis in *C. elegans* that examined developmentally programmed, somatic cell deaths, DNA damage responses are best studied in the germ line, which is the only proliferative tissue in the adult worm. The germ line proliferates both during larval development and adulthood, and comprises about half of the cell nuclei in the adult worm ***(2)***. The adult hermaphrodite gonad consists of two separate arms, each with a tubular structure. Throughout most of the length of each gonad arm, germ cell nuclei are present in a monolayer at the periphery of this tube, partially separated from each other by plasma membranes but retaining access to a common syncytial cytoplasmic core known as the rachis ***(2)***. Further, germ cells are organized in a temporal/spatial gradient along the distal-proximal axis (**Fig. 1**). The most distal end of the germ line contains a mitotic stem cell compartment, which is followed by nuclei in premeiotic S phase and progressively later substages of meiotic prophase; the most abundant group of meiotic cells are in the pachytene stage, during which homologous chromosomes are fully aligned and synapsed. The simultaneous presence of germ cells at different cell cycle and developmental stages indicates that adjacent germ cells are substantially insulated from their neighbors despite the syncytial organization of the germ line ***(2)***.

1.2. Programmed Cell Death in the C. elegans *Germ Line*

Two key findings paved the way for using the nematode germ line to investigate DNA damage checkpoint responses. The first crucial observation was that programmed cell death in *C. elegans* occurs not only in the developmentally determined somatic cell lineages, but also in the germ lines of hermaphrodite worms ***(3)***. Gumienny et al. noticed the presence of approx 0–4 germ cell corpses at any given time in the late pachytene region in the germ lines of normal adult hermaphrodites ***(3)***. The first morphologically visible step of programmed cell death in the germ line is the complete cellularization of the cell that is destined to die; other stages closely resemble programmed cell deaths that occur during somatic development.

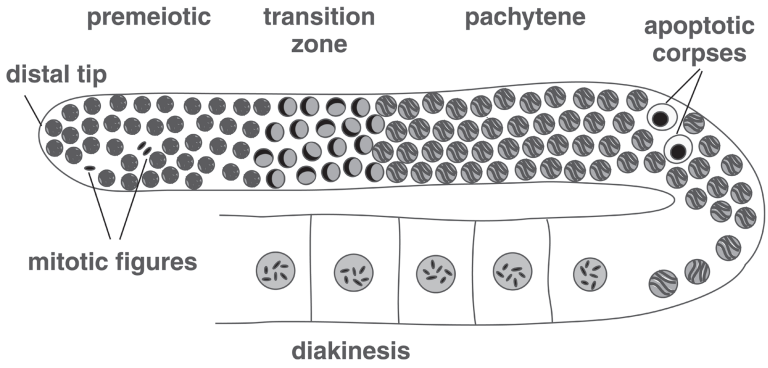

Fig. 1. Spatial organization of the *C. elegans* adult hermaphrodite germ line. Diagram shows one gonad arm, with individual germ line nuclei represented by black and gray circles; black areas represent the approximate appearance of chromatin as visualized by DAPI staining. The region adjacent to the distal tip contains premeiotic germ line nuclei, most of which are continuing to undergo mitotic cell cycles (mitotic figures are indicated); nuclei just distal to the "transition zone" region are in premeiotic S phase. Nuclei enter meiotic prophase in the transition zone, where a major nuclear reorganization that coincides with homologous chromosome pairing results in clustering of chromosomes toward one side of the nucleus; this organization imparts a crescent-shaped appearance to the DAPI-stained chromatin. The transition zone is followed by nuclei at the pachytene stage of meiotic prophase, in which chromosomes are organized in parallel pairs and are widely dispersed about the periphery of each nucleus. Apoptotic cell corpses (indicated by larger ovals with compact DAPI signals) are typically located in the late pachytene region, near to the bend of the gonad arm. Oocyte nuclei in diakinesis, the last stage of meiotic prophase, have greatly enlarged nuclei, and chromosome pairs have become highly condensed.

Within the germ line, only cells in the late pachytene stage of oocyte meiotic prophase are competent to die by apoptosis. Because dead cells are removed by phagocytosis, only a small number of dying cells or cell corpses will be visible at any given time; however, it has been estimated that as many as half of all potential oocyte precursors may be eliminated by programmed cell death during the reproductive life of an adult hermaphrodite. Since these deaths occur apparently independently of environmental stimuli, they have been termed "physiological" germ cell deaths. Similar to somatic apoptosis, physiological germ cell death is dependent on *ced-3* and *ced-4*, which encode the homologs of mammalian caspases and Apaf-1, respectively, and is suppressed by a gain-of-function mutation in Bcl2 ortholog *ced-9*. Further, the engulfment of germ cell corpses requires the same machinery used for engulfment during somatic

cell death. However, the somatic cell death trigger *egl-1* is not required for physiological germ cell death ***(3)***.

1.3. Germ Line Responses to Ionizing Radiation

A second key finding was that genotoxic stresses such as ionizing radiation (IR) can induce elevated levels of programmed cell death in the *C. elegans* germ line ***(1)***. Upon irradiation, *C. elegans* germ cells activate checkpoint pathways that lead to either a transient cell cycle arrest or to programmed cell death (**Figs. 2** and **3**). These two DNA damage responses are spatially separated within the gonad: whereas germ cells in the mitotic region halt cell cycle progression, germ cells in the late pachytene region undergo apoptosis. Cells outside the germ line show neither of these responses ***(1)***.

As is the case for physiological germ cell death, radiation-induced apoptosis appears to be restricted to female germ cells, requires *ced-3* (caspase) and *ced-4 (Apaf4)*, and is suppressed by a *ced-9 (Bcl2)* gain-of-function mutation ***(1)***. Unlike physiological germ cell deaths, however, radiation-induced apoptosis is partially dependent on *egl-1*. The DNA-damage checkpoint can be activated not only by exogenous genotoxic insults, but also by meiotic defects that result in accumulation of unrepaired meiotic recombination intermediates. As part of the normal meiotic program, double-strand DNA breaks (DSBs) are generated by the meiotic endonuclease SPO-11 to initiate meiotic recombination *(4)*. These DSBs are resected to generate single-strand 3' overhangs, which invade the DNA duplex on the homolgous chromosome via a reaction mediated by the conserved RAD-51 strand-exchange protein. Worms lacking RAD-51 are thought to accumulate unrepaired resected DSBs, which are recognized at least in part by the very same checkpoint pathways that sense radiation-induced DSBs, thereby triggering elevated levels of germ cell apoptosis ***(1,5)***.

In the mitotically proliferating region of the germ line, a transient halt in cell cycle progression is the second defining output of checkpoint activation ***(1)***. Under normal growth conditions, the total number of syncytial germ cell nuclei increases steadily over time. After ionizing irradiation, however, the number of germ cell nuclei does not increase over a time window of 12 h as cell proliferation is transiently halted (**Fig. 3**). In addition to arrest of cell proliferation, the volume of mitotic germ cell nuclei as well as their surrounding cytoplasm becomes greatly enlarged following irradiation (**Fig. 3**), presumably because cellular and nuclear growth continues during radiation-induced cell-proliferation arrest. This phenomenon parallels classical descriptions of cell cycle arrest phenotypes in yeast and *Drosophila* systems ***(6,7)***.

1.4. Germ Line Responses to Replication Block

Hydroxyurea (HU) depletes cellular dNTP pools through its specific inhibition of ribonucleotide reductase, and thus has been widely used as a potent

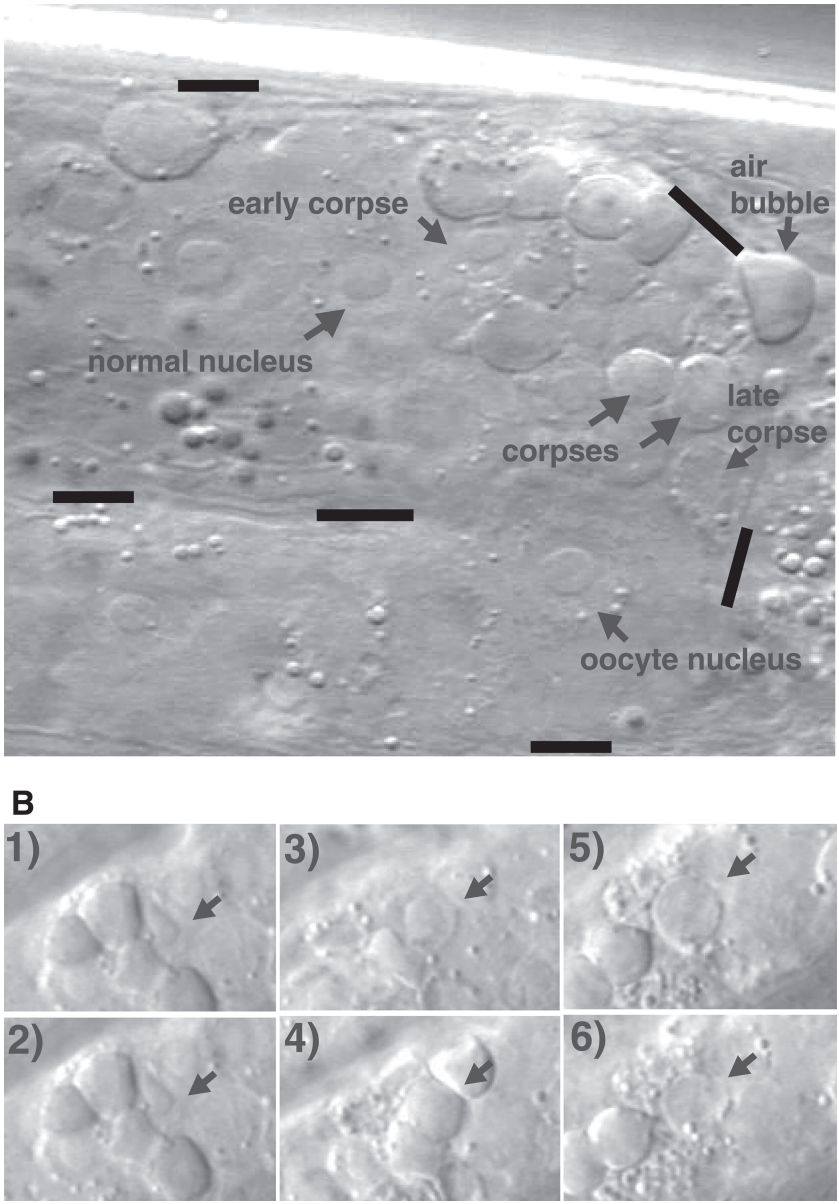

Fig. 2. Morphology of apototic germ cell corpses. **(A)** Example of massive germ cell death at the bend of the germ line. The morphologies of normal pachytene-stage meiotic prophase nuclei and of "early corpses," "corpses," and "late corpses" are indicated by arrows. **(B)** Time course of programmed germ cell death, following the fate of a dying cell over a period of approx 1.5 h. The arrow in each panel indicates the same cell at progressively later time points. Adapted from ***(1)***.

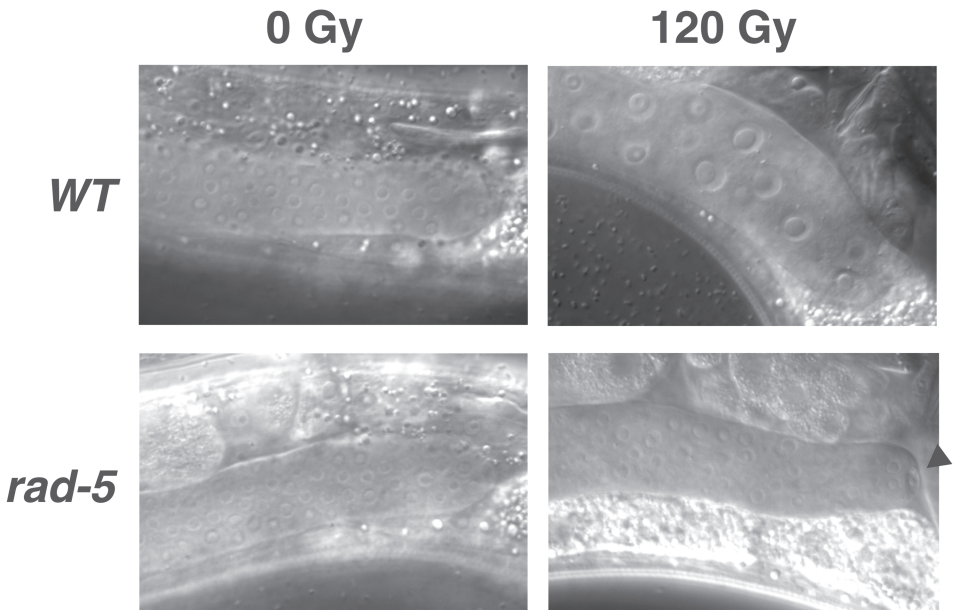

Fig. 3. Proliferation arrest phenotype of mitotic germ cells in response to checkpoint activation by IR, viewed with Nomarski microscopy. Note the enlargement of nuclei and the surrounding cytoplasm in response to irradiation in the WT animal. Checkpoint-defective animals like *rad-5* worms do not respond to ionizing irradiation. The arrowhead points to the distal tip cell.

indirect inhibitor of DNA synthesis. Numerous studies using HU to inhibit DNA replication have uncovered evidence for a checkpoint that monitors S phase progression in eukaryotic cells ***(8–10)***. Not surprisingly, chronic exposure of mature *C. elegans* hermaphrodites to HU elicits a block to germ cell proliferation, presumably as a consequence of inhibiting DNA replication ***(11)***. Compared with untreated control germ lines, HU-treated germ lines that have been stained with DAPI to label DNA (1) lack condensed mitotic figures representing nuclei undergoing M phase of the cell cycle; and (2) contain a reduced density of oversized nuclei with abnormally diffuse DAPI signals in their premeiotic regions **(Fig. 4B)**. Both in DAPI-stained preparations and when viewed

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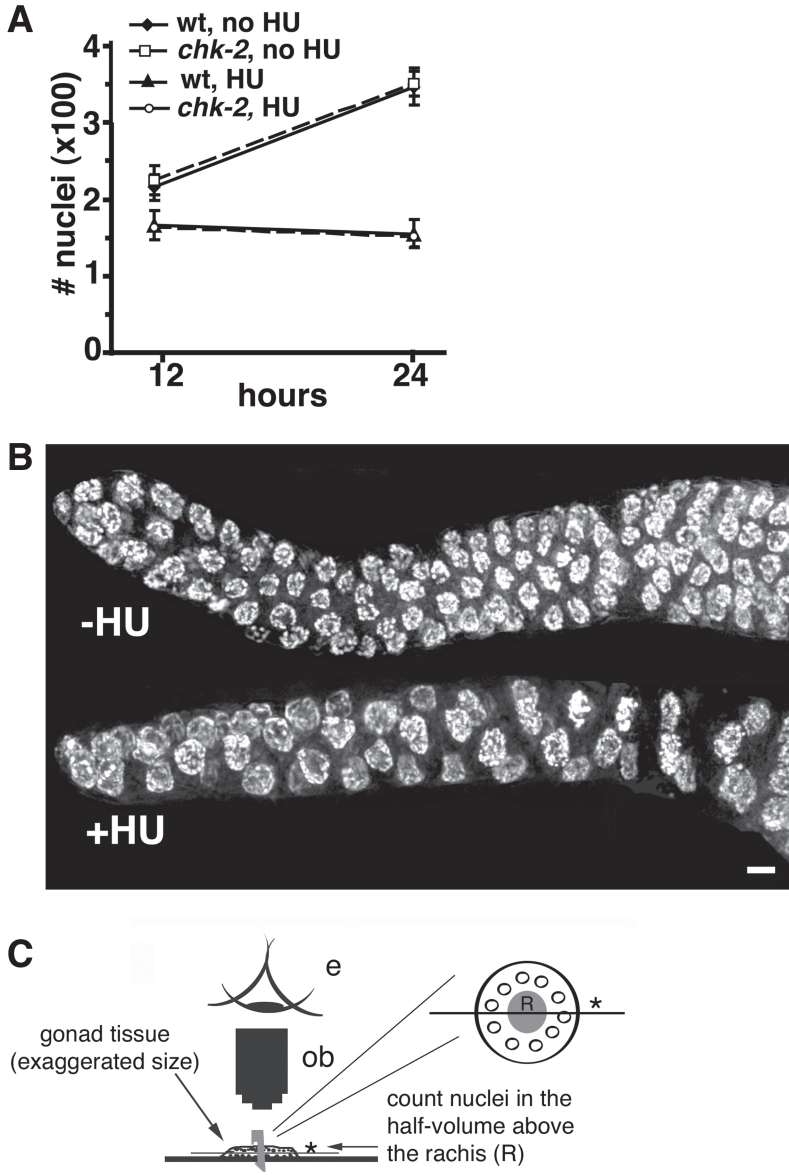
Fig. 4. *(opposite page)* Assessment of HU-induced germ cell proliferation arrest. **(A)** Quantitation of numbers of germ cell nuclei in wild-type and *chk-2* mutant worms chronically exposed to HU, compared with untreated controls. Germ line nuclei in "optically bisected" gonad arms were counted 12 or 24 h after initiating the exposure of L4 larvae to 25 m*M* HU. Each data point represents the average value from 4 to 10 germ lines; error bars indicate standard deviations. Whereas germ cell numbers increased substantially between the 12- and 24-h time points in untreated control germ lines, no increase in numbers was observed in HU-treated germ lines, indicating arrest



of germ cell proliferation; this checkpoint response was not abrogated in the *chk-2* mutant. **(B)** Morphological changes in premeiotic nuclei of *chk-2* mutants after 12 h of HU exposure; the response of wild-type germ lines is identical. Shown is the distal germ line region, which primarily contains premeiotic nuclei. In HU-treated germ lines, nuclei are substantially enlarged, reduced in number, and DAPI signals appear abnormally diffuse. Bar, 4 μm. **(A)** and **(B)** are reprinted with permission from ***(11)***. **(C)** Diagram illustrating "optical bisection" of the germ line. The nucleus-free core (rachis) of the germ line is depicted in gray in the enlarged cross-section (R). The straight line bisecting the germ line at the midpoint along the Z axis represents the lower boundary for counting; nuclei in the half-volume of the germ line at or above this line should be included in counts. (e), observer eye, (ob), objective lens.

by Nomarski microscopy in live animals, the appearance of nuclei in the premeiotic region of HU-treated germ lines is reminiscent of that exhibited by premeiotic nuclei arrested by ionizing radiation (***[12]*** and AJM, unpublished results). Germ line nuclei already in meiotic prophase appear largely unaffected by HU treatment.

HU-induced cell cycle arrest in *C. elegans* germ line nuclei appears to be reversible in a majority of affected germ cells, since mitotic figures reappear and many premeiotic nuclei exhibit normal size and morphology by 24 h after removal from HU (AJM, unpublished). Even 36 h following removal from HU, however, a handful of nuclei continue to exhibit arrest morphology. The apparent inability of a subset of nuclei to recover from arrest might reflect general cellular toxicity of the HU treatment, or it might reflect a difference between nuclei that were in S phase vs other phases of the cell cycle when dNTPs were depleted below critical levels. Alternatively, there might be differences in recovery potential between nuclei that were in pre-meiotic S phase at the time of depletion and those still undergoing proliferative cell cycles.

1.5. C. elegans *Checkpoint Genes and Checkpoint Responses*

Screens for *C. elegans* mutants defective in DNA-damage-induced apoptosis lead to the identification of *mrt-2, rad-5,* and *hus-1* as genes encoding potential checkpoint components ***(1,12,13)***. Mutations in these genes result in abrogation of IR-induced cell-cycle arrest and resistance to IR-induced apoptosis, and render the worms hypersensitive to DNA damage. *mrt-2* was found not only to be required for the DNA-damage checkpoint but also for the regulation of telomere replication ***(14)***. Positional cloning revealed that *mrt-2* encodes the worm ortholog of the *Schizzosaccharomyces pombe rad1* and *Saccharomyces cerevisiae rad17* checkpoint genes. *rad1*/*rad17* has previously been shown to be involved in yeast DNA damage checkpoints ***(14)***. The demonstrated role of this conserved checkpoint gene in IR-induced proliferation arrest and apoptosis confirmed the previously tentative conclusion that these are indeed bona fide checkpoint responses. Further, this result prompted experiments testing whether similar checkpoint defects could be elicited by RNAi of the *C. elegans* orthologs of other known yeast checkpoint genes. It was found that RNAi for worm orthologs of mammalian ATM and ATR, Rad-17, and p53bp1 indeed abrogated IR-induced proliferation arrest, further confirming the operation of conserved checkpoint pathways in this response ***(13)***. However, defects in IR-induced apoptosis could be elicited only at a low penetrance, most likely because these checkpoint genes could only be partially inhibited in meiotic pachytene cells by RNAi ***(13)***.

rad-5 was the first conserved checkpoint gene whose function in a DNA damage checkpoint was defined in the *C. elegans* system. *rad-5* is an essential

gene, and the two known *rad-5* mutations result in temperature-sensitive lethality ***(12)***. Cloning of *C. elegans rad-5* revealed that this gene is related to *S. cerevisiae TEL-2*, an essential gene shown to be involved in telomere-length regulation ***(15)***.

HU-induced cell cycle arrest in the proliferating population of *C. elegans* germ cells also appears to be a bona fide checkpoint response, since the arrest is abrogated in the *rad-5* mutants ***(12)***. The checkpoint pathway triggered by HU treatment is distinct from that triggered by IR treatment, however, since neither *mrt-2* nor *hus-1* appears to be required for HU-induced arrest.

In addition to checkpoint genes involved in both IR-induced proliferation arrest and cell death responses, one gene product has been demonstrated to play a role in triggering IR-induced apoptosis yet appears to be expendable for the proliferation-arrest response. Although it initially evaded detection by conventional homology searches, bioinformatics approaches using generalized profiles revealed that the worm genome encodes a distant homolog of the mammalian p53 tumor ssuppressor gene, termed *cep-1* (*C. elegans* p53-like) ***(16,17)***. Sequence alignments revealed that many of the p53 residues implicated either in DNA binding or in oncogenesis are conserved in *cep-1*. Unlike mammalian p53 but similar to *Drosophila* p53, *cep-1* is not required for DNA damage-induced proliferation arrest ***(16,17)***. The transcriptional activation of the *cep-1* target gene *egl-1* contributes in part to IR-induced germ cell death; thus the transcriptional induction of *egl-1* can also serve as a marker for DNA-damage checkpoint activation ***(18)***.

2. Materials

1. M9 buffer: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, H_2O to 1 L, 1 m*M* $MgSO_4$ (added after sterilization); pH should be between 6.9 and 7.0.
2. NGM agar: 3 g NaCl, 17 g agar, 2.5 g peptone, 1 mL cholesterol (5 mg/mL in EtOH), 975 mL H_2O. Autoclave, and then add the following sterile solutions, mixing after each addition: 1 mL 1 *M* $CaCl_2$, 1 mL 1 *M* $MgSO_4$, 25 mL 1 *M* potassium phosphate (pH 6.0). Pour plates. Store in plastic boxes with covers at room temperature for a couple of days before use to allow the plates to dry.
3. 1X egg salts buffer: 118 m*M* NaCl, 48 m*M* KCl, 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 5 m*M* HEPES (pH 7.4).
4. PBT: 1X PBS (137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4) plus 0.1% Tween-20.

3. Methods

3.1. Checkpoint-Mediated Germ Cell Apoptosis

The DNA damage checkpoint response during meiotic prophase is measured by scoring numbers of apoptotic germ cell corpses in the meiotic prophase

region of the adult hermaphrodite germ line. Checkpoint mutants exhibit less germ cell death than wild-type worms, whereas repair mutants tend to exhibit increased levels of germ cell death ***(1)***. *C. elegans* germ cell deaths are similar to the programmed cell deaths that occur during somatic development of the nematode, and can be readily observed in living animals using standard Nomarski differential interference contrast (DIC) microscopy. Live worms are mounted for microscopy as follows (adapted from ***19***).

3.2. Preparing Slides for Nomarski Microscopy

Prepare an agar pad as follows:

1. Place a slide between two spacer slides onto which you have put two layers of lab tape.
2. Put a few drops of hot 4% agar (in H_2O) on the slide.
3. Rapidly cover the hot agar with another slide and press down.
4. Let the agar solidify, then slide the top slide off the pad. Don't try to lift it off, but rather slide it off laterally.
5. Cut off excess agar on sides with razor blade.
6. Add 5 µL of 30 m*M* sodium azide in M9 buffer.
7. Add 5–30 worms.
8. Add coverslip.
9. Observe apoptotic corpses.

3.3. Morphology and Identification of Apoptotic Corpses in the C. elegans *Germ Line*

The method for quantifying the apoptosis response of the DNA damage checkpoint that requires the fewest experimental manipulations is direct observation in live animals by Nomarski microscopy. In order to use this approach, it is necessary to learn to identify apoptotic corpses in the germ line ***(1)***. Scoring is aided by the fact that germ cell apoptosis occurs mostly during the late pachytene stage, mainly at the bend region of the germ line (**Figs. 1** and **2**).

The first morphological sign of impending germ cell death is a decrease in the refractivity of the cytoplasm that occurs concomitant with an increase in refractivity of the nucleus (**Fig. 2B,** parts 1 and 2). In addition, a distinct boundary between dying cells and the surrounding germ line becomes visible (**Fig. 2A** "early corpse," and **Fig. 2B,** part 3); soon thereafter, both nucleus and cytoplasm become increasingly refractile and start to blend with each other until they resemble a flat, round, highly refractile disk (**Fig. 2A** "corpse," and **Fig. 2B,** part 4). After about 10–30 min, this flat disk often gets distorted and finally starts to disappear (**Fig. 2B,** parts 5 and 6). In late-stage corpses, the nucleus of the dying cell decreases in refractility, begins to appear crumpled, and finally vanishes within less than 1 h (**Fig. 2B,** parts 5 and 6). Late corpses often accu-

mulate granular structures at their rim (**Fig. 2A** "late corpse"). The morphology of corpses as well as the kinetics of their disappearance is similar between somatic and germ cell apoptosis. However, as germ cells are only partially surrounded by a plasma membrane, the first step in germ-cell death is the full cellularization of the apoptotic cell. Under conditions where massive germ-cell death occurs, corpses tend to accumulate next to each other **(Fig. 2A)**. Sometimes, when massive germ cell death occurs, "late apoptotic corpses" also accumulate at more proximal positions in the germ line and tend to align next to developing oocytes (*see* **Note 1**).

3.4. Visualization of Germ Cell Corpses by Acridine Orange Staining

An alternative means for assessing germ cell apoptosis takes advantage of the fact that apoptotic corpses can be stained in live animals by acridine orange (AO) and visualized by fluorescence microsopy. Although this approach requires additional manipulation of the samples compared with Nomarski microscopy, it is preferred by some investigators.

1. Use 5 μL of AO stock (10 mg/mL) per mL of M9 as a staining solution; AO is light sensitive, so stock solution should be stored in dark tubes (AO: Molecular Probes Inc. A3568).
2. Add 0.5 mL of staining solution to 60-mm plate of worms.
3. Rotate plates to make sure that the staining solution is distributed evenly, and store plates in the dark for approx 1 h at RT.
4. Wash worms off plate with 1.5 mL M9 and transfer to a 1.5 mL tube. Spin worms down for 2 s at approx 800*g*; remove supernatant and wash three times in M9 by spinning for 2 s at 800*g* and removing the supernatant by aspiration.
5. Replate washed worms on new 60-mm plate and keep in the dark for approx 45 min.
6. Score worms with a fluorescence microscope within 1 h for the presence of fluorescent bodies indicative of apoptosis.

3.5. Regimens for Quantifying IR-Induced Germ Cell Death

To quantify apoptosis in response to IR treatment, the number of germ cell corpses is assessed following a range of radiation doses, at a range of time points following treatment. Two different regimens can be used; a most thorough investigation of potential mutants will employ both strategies. For both regimens, it is important that worms be closely age matched, which is accomplished by selecting hermaphrodite worms at the late L4 larval stage.

1. Irradiate late L4 animals with 0, 30, 60, and 90 Gy; score apoptosis after 12, 24, and 36 h (*see* **Note 2**).
2. Pick late-L4 animals, age 24 h, then irradiate with 0, 60, and 120 Gy; score apoptosis 2, 4, 6, and 12 h after irradiation (*see* **Note 3**)

Approx 15 germ lines should be scored for each time point and for each radiation dose. When different strains are to be compared, it is important to assess whether germ lines are of approximately equal size and proliferate similarly; in practice, egg-laying rates can be measured and used as a very rough surrogate for proliferation behavior (which is difficult to assess directly).

3.6. Assessing Checkpoint Activation by Scoring for Induction of egl-1

3.6.1. Transcripts

Induction of transcripts from the pro-apoptotic gene *egl-1* can also be used as an indicator of checkpoint activation ***(18)***.

1. Synchronize worms and irradiate 24 h after the L4 stage.
2. Isolate total RNA 0.5–36 h postirradiation with RNAzol B (AMS Biotechnology) according to the manufacturer's protocol, treate with DNAseI, and further purify using the Rneasy kit (Qiagen).
3. For cDNA synthesis, reverse-transcribe purified total RNA with 250U of MultiScribe Reverse Transcriptase (Applied Biosystems) using random hexamer primers.
4. Estimate relative amounts of *egl-1* cDNA by quantitative PCR in an ABI Prism 7700 sequence detector system. For *egl-1* amplification, use the following two primers: 5'-CAGGACTTCTCCTCGTGTGAAGATTC-3' and 5'-GAAGTCATC GCACATTGCTGCTA-3', which span the single *egl-1* intron ***(18)***. As an alternative to quantitative PCR, induction of *egl-1* transcription can be assessed qualitatively by using an *egl-1* GFP reporter***(18)***.

3.7. Assessing Mitotic Germ Cell Cycle Arrest Upon Ionizing Irradiation

1. To assess the proliferation arrest response to IR, worms are irradiated with 0–120 Gy at the late L4 larval stage.
2. 12 h postirradiation, mount worms for Nomarski microscopy and score the distal region of the germ line for the presence of sparsely spaced, enlarged nuclei.
3. Count the number of nuclei within a defined field ***(1,12)***. The defined field used in several published studies corresponds to an area 3.125 μm × 6.25 μm in the most distal (premeiotic) region of the germ line **(Fig. 3)**; germ-cell nuclei in all focal planes are counted. Using this approach, <5 germ lines should be scored for each genotype and dose tested (*see* **Note 4**).

3.8. Assessing HU-Induced Germ Cell Proliferation Arrest

1. Prepare fresh plates (one plate/genotype assayed) for each chronic exposure experiment.
2. On standard 60 × 15 mm worm culture plates containing approx 12 mL of NGM agar ***(20)***, spread a lawn of *E. coli* OP50 that fills most of the plate area. Prepare plates at least 2 d prior to applying HU (hydroxyurea), and let them dry on a benchtop (not in a humid box) at room temperature. For negative control, prepare plates without HU.

3. Overlay each plate with 250 µL of a solution containing 92 mg/mL HU (Sigma) in M9. Cover plates with their lids and allow solution to soak into plates overnight; use plates within 1 d (*see* **Note 5**).
4. Pick and add at least 40 L4 larvae of a given genotype to a single, freshly prepared HU plate. (As several worms in each experiment may die owing to a low level of general toxicity of HU, it is important to plate a number of worms in excess of the number to be dissected.) For controls, add L4 larvae of each genotype to non-HU-treated plates.
5. At 12 and 24 h following plating of worms on HU and control plates, prepare worms for DAPI staining. Transfer 10–20 worms into 30µL of 1X egg salts buffer on a 22 × 40 mm coverslip; sodium azide (15 m*M*) can be used as an anesthetic to immobilize worms, if desired. Use a single or a pair of 25-gage needles to quickly nick the worm, either in the vicinity of the posterior bulb of the pharynx, or in the vicinity of the anus. A successful nick will result in extrusion of one of the two gonad arms. Often a nick will only partially release the gonad arm; since full gonad release is required for the method of quantifying germ cell proliferation described below, at least five animals with a fully released gonad arm are required. Dissections must be performed quickly; dissected worms should spend no longer than 5 min in buffer prior to fixation.
6. Allow worms to settle briefly and carefully remove 15 µL of the buffer. Add 15 µL of fixation solution (7.4% formaldehyde [diluted freshly from a 37% solution] in 1X egg salts). Incubate dissected worms in fixative for 5–10 min.
7. Carefully remove 15 µL of the liquid, and sandwich worms/buffer/fix between the coverslip and a slide. SuperFrost Plus slides (Fisher) or lysine-coated slides should be used, as the internal tissue released from the worms sticks best to positively charged slides. Immediately immerse the slide, coverslip side up, in liquid nitrogen to freeze. Alternatively, place the slide on an aluminum block precooled to –70°C on dry ice. When frozen (approx 20 s), crack the coverslip off with a single downward slice of a razorblade situated between the edge of the coverslip and the slide itself. Immediately transfer the slide to 95% ethanol, prechilled to –20°C. Slides can be stored in this condition for at least 1 wk.
8. Warm slides to room temperature, then rehydrate through a series of 3-min PBT/ethanol washes containing increasing levels of PBT (1X PBS plus 0.1% Tween-20): for example, 25:75, 50:50, then 75:25 (PBT: 95% ethanol) followed by three washes in 100% PBT.
9. Incubate in 2 µg/mL 4', 6-diamidino-2-phenylindole (DAPI) for 5 min, then rinse five times for 5 min in PBT. Hold in the dark for 30 min to 1 h at 4°C in PBT; mount in 60% glycerol or alternative aqueous mounting media.
10. For scoring select only intact, fully released germ lines for analysis.
11. For consistent quantitation, germ lines should be "optically bisected" along the longitudinal axis of the gonad. Focus the microsope up and down through the Z-axis to reveal the rachis, the nucleus-deficient central cytoplasmic core of the germ line; the position of the rachis defines the bisection boundary. As it is often difficult to resolve nuclei on the side of the rachis farthest from the objective lens, it is best to restrict counts to nuclei in the half-volume of the germ line that

is closest to the objective lens **(Fig. 4C)**. In the X and Y dimensions, count nuclei from the distal tip through the end of the pachytene region of the germ line, ending at the point where nuclei expand markedly in volume as chromosome condensation increases, reflecting transition into the morphologically distinct diakinesis stage of meiotic prophase (during which six separate highly condensed DAPI-stained bodies can be resolved in each nucleus). If properly age-matched, individual control animals should exhibit little variation (approx 15%) in the number of nuclei per gonad arm (e.g., **Fig. 4A**) (*see* **Note 6**).

3.9. Scoring for Radiation Sensitivity (Rad Assay)

Germ lines of worms defective either in checkpoint pathways or repair pathways typically exhibit hypersensitivity to IR. One easily assayed manifestation of such sensitivity is a severe drop in the production of viable progeny following genotoxic insult; this can be reflected in a drop on the number of zygotes produced, the fractional viability of the zygotes produced, or both. To enhance the accuracy of the assay the experiment is done in duplicates.

1. Irradiate late L4 larval worms with 0 Gy, 30 Gy, 60 Gy, and 120 Gy.
2. 24 h later, when the germ line is already fully developed, place five worms on worm plates that contain a freshly seeded bacterial lawn approx 1 cm in diameter at the center of the plate.
3. Remove adult worms from plates after 10–12 h, and determine the percentage of hatched embryos 24 to 36 h later.
4. Determine, the rate of egg-laying per worm per hour by adding the number of dead embryos and hatched larvae.

In a typical experiment, the lethality of wild-type animals is approx 30% and 70% after irradiation with 60 and 120 Gy, respectively.

3.10. Using RNAi and Co-Suppression to Inhibit the Function of Candidate Checkpoint Genes

Whereas detailed analysis of the role of a gene involved in the DNA damage response is best conducted using a permanent chromosomal mutation, in practice the potential involvement of candidate checkpoint genes will most often be assessed initially using posttranscriptional gene silencing (PTGS) methods to inhibit candidate gene function. Two approaches are available for use in the *C. elegans* germ line: RNAi methods, which deliver dsRNA molecules that direct the degradation of corresponding target mRNAs ***(21)***, and transgene-mediated co-suppression, whereby a high-copy transgene array elicits PTGS of the corresponding endogenous gene ***(22,23)***. (In the latter method, transgenes are *not* designed explicitly to express dsRNAs; the mechanisms of RNAi and cosuppression appear to be mechanistically related in that they have overlapping genetic requirements, but there are also distinctions.) Empirically, we have

found that some genes respond more robustly to RNAi whereas others respond more robustly to co-suppression ***(13)***; because the reasons for this are obscure at present, researchers particularly interested investigating the roles of a few specific genes are encouraged to try both approaches.

3.10.1. RNAi of Checkpoint Genes

dsRNA can be delivered to *C. elegans* by a variety of routes, including injection, soaking, and feeding. For the RNAi injection and soaking procedures, dsRNAs are produced by in vitro transcription and annealing of the complementary RNA strands. In the injection procedure, dsRNA is injected into the gonad or into the gut of adult worms ***(21)***. In the soaking procedure, worms are incubated in a solution of dsRNA ***(24)***. In the RNAi feeding procedure, the gene to be inactivated is cloned into an *E. coli* vector that allows for the inducible transcription of both DNA strands ***(25)***. In practice, we have found that the delivery of RNAi by feeding has turned out to be the most successful technique for inactivating DNA damage checkpoint genes (Anton Gartner, unpublished observation). Further, it is easier to block IR-induced proliferation arrest than to block IR-induced apoptosis.

1. For RNAi by feeding, clone a cDNA or approx 1 kb of an exon-rich sequence into the L4440 RNAi feeding vector, which allows for inducible transcription of both strands of the insert, and transform into the HT115 *E. coli* strain.
2. Spread transformed *E. coli* on an LB amp plate overnight (2–3 cm^2). Resuspend the *E. coli* lawn in 200 μL LB, and use 50 μL of the resuspended culture to seed a NGM Amp (100 μL/mL) plate containing 6 m*M* IPTG.
3. Add, after a plate has dried, approx 3 P0 worms to the plate and incubate at 15°C for 3–4 d.
4. Three F1 worms (at the early L4 stage) are transferred, individually, each to its own plate freshly seeded by bacteria, and allowed to lay eggs for approx 24 h.
5. F1 worms are then removed and F2 worms are allowed to grow up to the L4 stage, treated with IR, and analyzed for radiation-induced cell cycle arrest as described above.

3.10.2. Co-Suppression of Checkpoint Genes

1. For checkpoint gene inactivation by co-suppression, amplify the promoter sequence and the first two exons of a target gene by PCR.
2. Phenol-chloroform extract PCR product and *rol-6* transformation marker ***(23)***.
3. Coinject 50 ng/μL PCR product with an equal concentration of *rol-6* transformation marker ***(23)***.
4. Select stable transformants according to standard procedures in the next two generations, and animals harboring the transgene array are analyzed for checkpoint responses as described above.

4. Notes

1. For quantification purposes, one does not follow the appearance and disappearance of corpses, but instead records a "snapshot" of the number of corpses present at a given time in each scored germ line (see below for dose/timing regimens). In practice, there is some subjectivity in scoring; whereas, the highly refractile corpses are readily recognized, "early corpses" and "late corpses" may not be recognized as consistently by all investigators. Thus, it is crucial that all genotypes, time points, and doses to be compared in a given analysis be scored by the same individual. Further, a novice in the field has to confirm that the structures being scored as corpses are indeed apoptotic corpses by verifying that they are not present in worms defective for the cell-death genes *ced-3* and *ced-4,* and/or that they stain with the dye acridine orange.
2. The advantage of this counting regimen is that it is easier to perform, and higher levels of germ-cell death are generally obtained. For example, upon irradiation with 120 Gy, an average of up to 25 corpses per germ line bend can be scored 36 h after irradiation. There is a potential complication in interpreting the results from this regimen, however. Whereas corpses scored 12 h after IR exposure (and likely most scored 24 h after exposure) would already have entered meiotic prophase at the time of irradiation, those scored at the 36 h timepoint would likely have been premeiotic at the time of exposure; these may have first undergone a transient cell cycle arrest, then recovered from arrest before entering meiotic prophase. Thus, differences in the kinetics of recovery, proliferation, or meiotic entry could potentially contribute to observed differences between strains in levels of programmed cell death.
3. Under this second counting regimen, it is clear that the programmed cell deaths scored directly reflect the response of cells that were already in the pachytene stage at the time of exposure. However, the numbers of apoptotic corpses detected are considerably lower when this regimen is employed. To sensitize detection of IR-induced cell death, germ cell apoptosis can be scored in strain backgrounds (e.g., *ced-1*) that are defective in the engulfment of apoptotic corpses; corpses persist for prolonged periods in such mutants, increasing the numbers of corpses that can be detected at any given time.
4. As an alternative to Nomarski, IR-induced proliferation arrest can also be visualized and quantified in fixed DAPI-stained germ lines as described below for the assessment of the response to HU-induced proliferation arrest. Although this approach requires several processing steps, it facilitates the scoring of experiments involving many samples, since fixation prevents asynchrony. Furthermore, the DAPI staining procedure permits detection of chromatin bridges and/or evidence for unequal mitotic chromosome segregation in mutants.
5. The final concentration of HU should be approx 25 m*M*. If plates contain more or less than 12 mL of NGM agar, adjust the amount of HU added accordingly.
6. If toxicity is observed with HU treatment, a lower concentration of HU (or a shorter exposure time) that still elicits arrest should be explored.

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