

DNA Strand Breaks in Mitotic Germ Cells of *Caenorhabditis elegans* Evaluated by Comet Assay

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DNA damage responses are important for the maintenance of genome stability and the survival of organisms. Such responses are activated in the presence of DNA damage and lead to cell cycle arrest, apoptosis, and DNA repair. In *Caenorhabditis elegans*, double-strand breaks induced by DNA damaging agents have been detected indirectly by antibodies against DSB recognizing proteins. In this study we used a comet assay to detect DNA strand breaks and to measure the elimination of DNA strand breaks in mitotic germline nuclei of *C. elegans*. We found that *C. elegans* *brc-1* mutants were more sensitive to ionizing radiation and camptothecin than the N2 wild-type strain and repaired DNA strand breaks less efficiently than N2. This study is the first demonstration of direct measurement of DNA strand breaks in mitotic germline nuclei of *C. elegans*. This newly developed assay can be applied to detect DNA strand breaks in different *C. elegans* mutants that are sensitive to DNA damaging agents.

INTRODUCTION

The integrity of DNA is continuously challenged by a variety of exogenous and endogenous DNA-damaging agents. Eukaryotes have evolved to possess a DNA damage response (DDR) that triggers cell cycle arrest, apoptosis, and DNA repair to protect the cell and ameliorate the threat to the organism.

The DDR signaling cascade is highly conserved in *Caenorhabditis elegans*. The DDR in the *C. elegans* germline involves the coordinated induction of DNA repair, cell cycle arrest, and apoptotic processes (Craig et al., 2012; Lemmens and Tijsterman, 2011). ATM-1 and ATL-1 are typical kinases of the DDR pathway that phosphorylate a large number of substrates in response to DNA damage (Garcia-Muse and Boulton, 2005; Lee et al., 2010). MRE-11, RAD-50, HUS-1, and MRT-2 proteins are required for the function of these kinases (Ahmed and

Hodgkin, 2000; Chin and Villeneuve, 2001; Hofmann et al., 2002). Through the activation of downstream kinases CHK-1 and CHK-2, signaling is amplified down to the activation of effector molecules (Kalogeropoulos et al., 2004; Lee et al., 2010; MacQueen and Villeneuve, 2001).

DNA double-strand breaks (DSBs) can be induced either directly by exposure to ionizing irradiation (IR) or indirectly by the topoisomerase I inhibitor camptothecin (CPT), which causes replication fork stalling and collapse in actively cycling cells. DDR proteins for DSBs in mammalian cells are the kinases ATM/ATR, the sensing complex MRE11-RAD50-NBS1, and CHK2 (Langerak and Russell, 2011). The presence of DSBs is indirectly detected by immunostaining the proteins γ -H2AX or RAD51 (Paull et al., 2000; Tarsounas et al., 2004). H2AX is phosphorylated (γ -H2AX) following exposure to IR and is densely localized around DSBs. Since RAD51 plays an essential role in homologous recombination for DSB repair in mammalian cells, RAD51 foci formation is thought to represent the presence of DSBs. Thus, immunostaining of γ -H2AX and RAD51 is useful for visualizing the localization of DSBs. RAD51 foci formation has also been used to detect the sites of DSBs following IR in *C. elegans* (Wicky et al., 2004).

Another method used to detect DSBs and their repair is the comet assay, a rapid and quantitative technique by which damaged or broken pieces of DNA are measured at the level of individual cells (Olive and Banath, 2006). Increased comet tails indicate the induction of DNA strand breaks. Initially, DNA strand breaks induced by IR or UV irradiation were detected in human blood cells by the comet assay (Lankinen et al., 1996; Olive et al., 1990), and DNA strand breaks induced by other agents such as CPT were subsequently detected (Godard et al., 2002). To date, there is only one report of use of the comet assay in *C. elegans* to investigate the effect of nicotine on cultured embryonic cells (Sobkowiak and Lesicki, 2009), but the authors did not investigate mitotic germline nuclei to observe DNA strand breaks induced by IR or CPT.

The identified *C. elegans* protein BRC-1 (C36A4.8), a protein of 596 amino acids, is an ortholog of human BRCA1. Studies of the effects of depletion of *brc-1* have described IR sensitivity and enhanced levels of germ cell death and DNA fragmentation after IR, suggesting that BRC-1 is involved in DSB repair by homologous recombination (HR) (Boulton et al., 2004). A role of BRC-1 in the repair pathway of inter-sister meiotic DSBs is also suggested (Adamo et al., 2008). However, the defect in DSB repair has not been well investigated at the DNA level. In this

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study, we used the comet assay to investigate DNA strand breakage and repair in the N2 wild-type strain and *brc-1* mutant which was chosen as a suitable DNA repair mutant.

MATERIAL AND METHODS

Strains

The *C. elegans* Bristol N2 wild-type strain was maintained on nematode growth media (NGM) plates at 20°C as described previously (Lee et al., 2010). The *C. elegans* mutant strain *brc-1(tm1145)* was obtained from the *Caenorhabditis* Genetics Center (USA).

Treatment with ionizing radiation and camptothecin

L4-stage animals on NGM plates were irradiated using a ¹³⁷Cs source (gamma cell 3000 ELAN) with a rate of 321 rad/min on ice. L4-stage animals were grown on NGM plates containing 0, 1, 2, 5, 10, 20, or 40 μM CPT (Sigma-Aldrich) for 24 h at 20°C.

Measurement of embryonic survival

IR-treated worms were allowed to lay eggs. After 24 h, the number of unhatched eggs was counted. L4-stage animals were grown on NGM plates containing 0, 1, 2, 5, 10, 20, or 40 μM CPT for 24 h and then transferred to CPT-free plates with *E. coli* OP50, where eggs were laid. After 24 h, the numbers of hatched and unhatched eggs were counted. The embryonic survival percentage was calculated by dividing the number of hatched eggs by the total number of eggs laid.

Comet assays

For the glyoxal-treated comet assay, mitotic germline nuclei were treated with glyoxal to denature the DNA (Hyun et al., 2008). The dissected gonads (30–40 worms) were cut to expose the mitotic tip regions. The mitotic compartments were mixed with glass beads (212–300 μm in diameter) in a microcentrifuge tube and disrupted in a mini bead-beater (company) at 500 x g for 10 s. The supernatants containing mitotic nuclei were separated by centrifugation at 100 x g for 10 s and mixed with low-melting point agarose. The mixture was loaded on an agar-coated microscope slide. The slide was immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl: pH 8.4, 1% Triton X-100, 10% DMSO) at 4°C for 15 min and then incubated with 1 M glyoxal in lysis buffer (1:1 ratio) at 4°C for 1 h. Electrophoresis was performed at 50 volts for 90 min in TBE buffer. After electrophoresis, the slides were washed with deionized H₂O and then stained with DAPI (4,6-diamidino-2-phenylindole, 1 μg/ml, Sigma-Aldrich) or SYBR Gold (1:10,000 dilution, Molecular Probes) for 10 min. Observations were performed under an epifluorescence microscope (Carl Zeiss Axioskop2 plus). Quantitation was performed on 60–100 nuclei from each gel using Comet Assay IV software (Perceptive Instruments, Ltd.).

Neutral comet assays were performed by a slight modification of the glyoxal-treated comet assay: after the lysis step, instead of glyoxal treatment, the slides were incubated with RNase A (1 mg/ml, Sigma-Aldrich) and proteinase K (32 U/ml, Roche) for 1 h at 37°C and then electrophoresis was performed at 20 volts for 90 min in TBE buffer. After electrophoresis, the slides were stained and analyzed as above. We used Olive tail moment in this study because it is considered independent of comet shape and is the best-established parameter for this assay, with less variability than tail length and tail DNA content.

Immunostaining

The gonads of IR-irradiated worm were dissected 1h after irradiation.

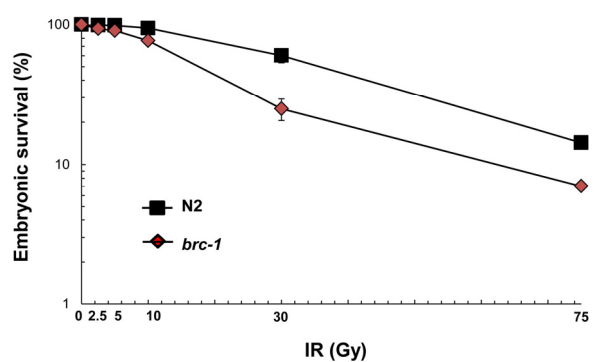


Fig. 1. The Percent survival of embryo survival after exposure to IR. N2 and *brc-1(tm1145)* mutants were irradiated at the indicated doses (0, 2.5, 5, 10, 30, 75 Gy) on ice and then transferred to NGM plates with *E. coli* OP50, where eggs were laid. Hatching percentages were measured. Error bars indicate SEM.

The gonads of CPT-treated worms were dissected 24 h after treatment. The gonads were extruded, fixed in 4% paraformaldehyde, and immunostained as described previously (Garcia-Muse and Boulton, 2005; Hyun et al., 2012). Briefly, the gonads were blocked with PBSBT (1X PBS, 0.5% bovine serum albumin, 0.1% Triton X-100) containing 2% non-fat milk at 4°C overnight. The gonads were incubated with primary antibody (1:1,000 dilution for anti-RAD-51, a polyclonal antibody generated with N-terminal 103 amino acids in rabbit) in humid chambers for 16 h at 4°C and then with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) for 1 h at room temperature in dark conditions. After staining with DAPI, the gonads were mounted on a 1% agarose pad and observed under an epifluorescence microscope (Carl Zeiss Axioskop2 plus).

RESULTS

Detection of DNA strand breaks induced by IR in *C. elegans*

Embryonic survival following ionizing radiation

Since *C. elegans brc-1(RNAi)* animals have been shown to be radiation sensitive (Boulton et al., 2004), we examined the sensitivity of *brc-1(tm1145)* mutants to IR. The mutants were treated with ¹³⁷Cs γ-rays at a dose of 0–75 Gy. The hatching percent of laid eggs from IR-treated worms decreased with increasing doses of IR, and the reduction in hatching percentage of *brc-1* mutants was greater than that of wild-type N2, indicating that the *brc-1* mutants are radiation sensitive (Fig. 1). At 30 Gy, N2 showed 60% survival whereas the *brc-1* mutant showed 25% survival (Fig. 1). This dose was chosen for subsequent experiments. Since the primary determinant of IR sensitivity is the efficiency of DNA double-strand break (DSB) repair, DNA strand breaks induced by IR and the DNA repair of DNA strand breaks were examined in *brc-1* mutants and wild-type N2.

DSBs accumulate in the *brc-1(tm1145)* mutant after IR

DNA repair defects have been observed in *brc-1(RNAi)* animals (Boulton et al., 2004) and the defect in *brc-1(tm1145)* mutants has been evaluated by the formation of nuclear RAD-51 foci after IR (Polanowska et al., 2006) since RAD-51 plays an essential role in homologous recombination (Alpi et al., 2003; Petalcorin et al., 2007). However, IR-induced DSBs and repair

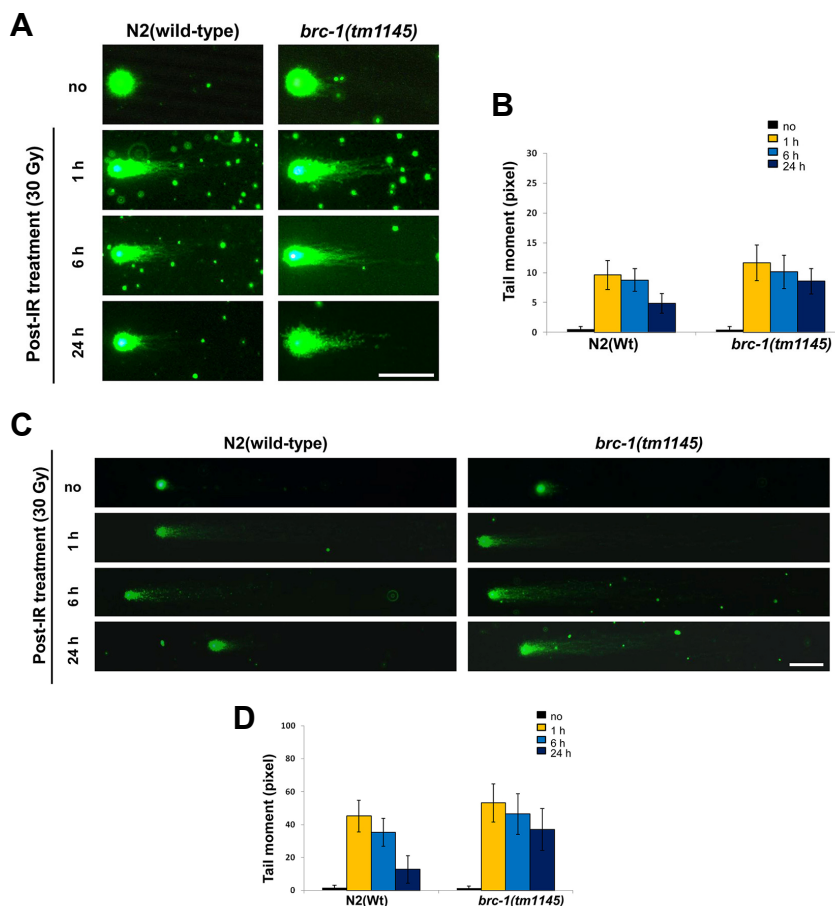


Fig. 2. Analysis of Comet assay. Worms were irradiated with 30 Gy of IR and postincubated for various recovery times. Comet slides were prepared, lysed, treated with glyoxal, and electrophoresed in a neutral buffer. (A) Comet images were captured using an epifluorescence microscope (Carl Zeiss), Scale bar, 50 μ m. (B) The tail moment of comets in (A) was analyzed using Comet Assay IV software. Comet slides were prepared, lysed, treated with RNase and proteinase K, and electrophoresed in a neutral buffer. (C) Comet images were captured using an epifluorescence microscope (Carl Zeiss), Scale bar, 50 μ m. (D) The tail moment of comets in (C) was analyzed using Comet Assay IV software.

kinetics in *brc-1* mutants have not been directly examined. We examined DNA strand breakage and repair using the comet assay, in which the extent of DNA strand breakage is assessed by DNA migration in the comet tail following irradiation with 30 Gy. Representative images of glyoxal comets are shown in Fig. 2A. Comet tails were observed at 1 h after IR, indicating that DNA strand breaks were induced by IR. The analysis of tail moments in 100 comets at recovery time of 24 h after IR revealed that 55% of the DNA strand breaks were repaired in N2, whereas only 27% of the DNA strand breaks were repaired in *brc-1* mutants (Fig. 2B).

The neutral comet assay was also performed to specifically examine DSB and repair. Comet tails were observed at 1 h after IR (Fig. 2C), indicating that DSBs were induced by IR. The analysis of tail moments in 100 comets at recovery time of 24 h after IR revealed that 73% of the DSBs were repaired in N2, compared with 30% in *brc-1* mutants (Fig. 2D).

The tail moments in two assays were different. The extent of repair of N2 measured by the glyoxal-comet assay (Figs. 2B and 2D) was lower than that by the neutral comet assay, indicating that unrepaired single-strand breaks reflect the difference. The extent of repair *brc-1* mutants at recovery time of 24 h is similar, indicating that unrepaired DSBs may reflect the extent of repair. Taken together, these data support a previous finding that *brc-1* mutants are defective in DSB repair (Boulton et al., 2004).

Detection of DNA strand breaks induced by camptothecin in *C. elegans*

Embryonic survival following camptothecin treatment

CPT, a selective inhibitor of topoisomerase I (TOP1), stabilizes TOP1-DNA covalent complexes. Collisions between the replication fork migrating along the DNA and a trapped TOP1-DNA covalent complex result in irreversible replication fork arrest and DSB formation at the fork (Pommier, 2006; Ryan et al., 1991). Since the sensitivity of *brc-1* mutants to CPT has not been reported, we first examined the embryonic survival of *brc-1* mutants after treatment with the indicated concentrations of CPT for 24 h (Fig. 3). The hatching percentage of laid eggs from the CPT-treated *brc-1* mutants was greatly reduced after CPT treatment. At 5 μ M CPT, the N2 strain showed 60% survival, compared with 22% for the *brc-1* mutant. We selected a concentration of 5 μ M CPT for the next experiments.

DSBs accumulate in the *brc-1(tm1145)* mutant after CPT treatment

We have previously shown that CPT induces DSBs in wild-type N2 by demonstrating an increase in the numbers of germline nucleus showing RAD-51-positive foci (manuscript in preparation). RAD-51 foci were also detected in mitotic nuclei of N2 and *brc-1* after CPT treatment (Fig. S2). We examined whether CPT-induced RAD-51 foci formation reflects DNA strand breaks

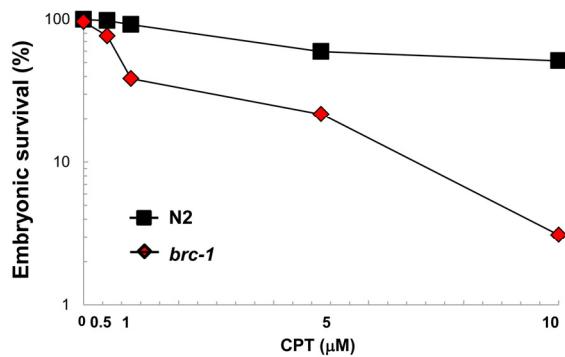


Fig. 3. The Percent survival of embryo survival after treatment with CPT. N2 and *bcr-1(tm1145)* mutants were treated with the indicated concentrations of CPT for 24 h and then transferred to CPT-free plates with *E. coli OP50*, where eggs were laid. Hatching percentages were measured. Error bars indicate SEM.

in germline nuclei in *C. elegans* and investigated the repair of CPT-induced DNA strand breaks using the comet assay.

The glyoxal comet assay was first performed to confirm the presence of DNA strand breaks. Representative images are shown (Fig. 4A). There was an increase in CPT-induced DNA strand breaks compared with non-damaged controls in both wild-type N2 and *bcr-1* mutants (compare the data of no (no CPT-treated worms) with the data of 0 h in Figs. 4A and 4B). The analysis of tail moments for DNA strand breaks observed at different recovery times after CPT treatment revealed that 60% of the DNA strand breaks were removed at 24 h after CPT treatment in N2, whereas the extent of DNA strand breakage remained unchanged in *bcr-1* mutants (Fig. 4B). The N2 worms showed little removal of DNA strand breaks before 12 h and seemed to start remove after that time. These results suggest that repair of CPT-induced DNA strand breaks is defective in *bcr-1* mutants.

The neutral comet assay was also performed to assess DSBs induced by CPT. Representative images are shown (Fig. 4C). There was an increase in CPT-induced DSBs compared with non-damaged controls in both wild-type N2 and *bcr-1* mutants (compare the data of no (no CPT-treated worm) with the data of 0 h in Figs. 4C and 4D). The analysis of tail moments in 100 comets of N2 at each time points after CPT treatment revealed that the tail lengths of DSBs were gradually decreased and greatly diminished (85%) at 24 h, but remained long (Fig. 4D) in *bcr-1* mutants.

The tail moments in two assays were different. The extent of repair of N2 measured by the glyoxal-comet assay (Figs. 4B and 4D) was lower than that by the neutral comet assay, indicating that unrepaired single-strand breaks reflect the difference. Interestingly, DSBs were further generated during recovery times in *bcr-1* mutants when treated with CTP. These data further indicate that *bcr-1* mutants are defective in DSB repair and implicate a role for BRC-1 in the DSB repair process.

DISCUSSION

In this study, we have investigated DNA DSB induction and repair in wild-type N2 and *bcr-1* mutants using the comet assay. Although both RAD-51 foci formation and chromosome fragmentation have been applied for the detection of DSB induction and DSB repair in mitotic germline nuclei *C. elegans*, use of the

comet assay has not previously been reported in those nuclei. In this study we successfully detected the extent of DNA strand breaks in *C. elegans* using the comet assay.

We chose a *bcr-1* mutant as a suitable DNA repair mutant for comparison of the detection of DNA strand breakage and repair with that of wild-type. BRC-1 was previously identified as the *C. elegans* ortholog of BRCA1, which is required for DSB repair in mammalian cells, and *bcr-1* depleted animals were shown to be sensitive to DSB-inducing IR, suggesting a role of BRC-1 in HR in *C. elegans* (Boulton et al., 2004). BRC-1 forms a complex with BRD-1 and is recruited to sites of DNA damage (Boulton et al., 2004; Polanowska et al., 2006), consistent with the role of BRCA1 in mammalian cells (Meza et al., 1999; Moynahan et al., 1999; Scully et al., 1999). BRC-1 is also required for HR between sister chromatids in meiotic cells (Adamo et al., 2008). In this study, we showed that *bcr-1* mutants were sensitive to IR and CPT treatments, suggesting that BRC-1 may be involved in processing DNA damage induced by these treatments.

The comet assay, also known as single-cell gel electrophoresis, can detect DNA damage and repair kinetics at the level of a single cell and has been shown to be a suitable tool for studying the induction and repair of radiation-induced DSBs (Olive and Banath, 2006). The alkaline comet, in which DNA is mobilized under alkaline conditions for DNA denaturation, detects both single-stranded DNA breaks and DSBs, without distinguishing between the two. The neutral comet, in which DNA migrates under neutral conditions, detects only DSBs.

There is only one previous report of use of the comet assay in *C. elegans*. Sobkowiak et al. used the alkaline comet assay to investigate the *in vitro* response of cultured embryonic cells of *C. elegans* to nicotine (Sobkowiak and Lesicki, 2009). Their study suggested that the comet assay could be performed with other cells from *C. elegans*; however, the authors did not investigate the comet assay using mitotic germline nuclei. In this study, we physically isolated mitotic germline nuclei of *C. elegans* as described in the Methods and successfully carried out comet assays.

We treated germline nuclei with glyoxal instead of alkaline treatment because the presence of alkali-labile sites in DNA can result in the introduction of additional single-strand breaks upon alkali treatment during DNA sample processing. Glyoxal is known to covalently bind to only solution-exposed guanine bases (Broude and Budowsky, 1973). We have previously used glyoxal in DNA hybridization of *C. elegans* genomic DNA (Hyun et al., 2008) because the *C. elegans* genomic DNA was detected to be degraded under alkaline electrophoresis condition (unpublished data). There was a clear difference in tail length between untreated germline nuclei from either N2 or *bcr-1* mutants and IR-treated germline nuclei, indicating that IR induces DNA strand breaks. CPT-induced DNA strand breaks were also detected in N2 and *bcr-1* mutants. Unchanged tail length of CPT-induced DNA strand breaks in *bcr-1* mutants may present that initial DNA strand breaks generated by CPT treatment were not removed and no more DNA strand break was produced during recovery time, indicating defects in repair of DNA strand breaks. Consistent with these findings, DNA strand breaks induced by IR or CPT can be detected by the alkaline comet assay or glyoxal comet assay in mammalian cells (Godard et al., 2002; Lankinen et al., 1996; Pachkowski and Nakamura, 2013).

To attain high enough sensitivity under the neutral comet assay, germline nuclei were treated with ribonuclease A and proteinase K to obtain relatively pure DNA free from RNA and most proteins (Singh and Khan, 1995). In the wild-type N2 strain,

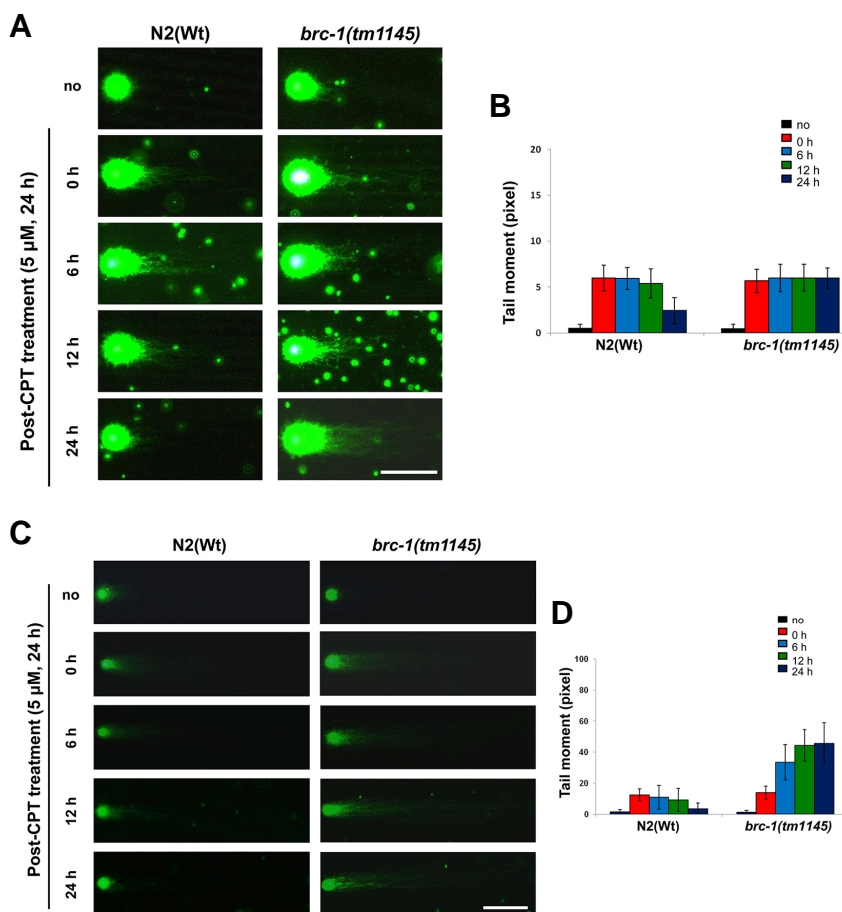


Fig. 4. Analysis of Comet assay. Worms were treated with 5 μ M CPT for 24 h and postincubated on new fresh NGM plates for various recovery times. Comet slides were prepared, lysed, treated with glyoxal, and electrophoresed in a neutral buffer. (A) Comet images were captured using an epifluorescence microscope (Carl Zeiss), Scale bar, 50 μ m. (B) The tail moment of comets in (A) was analyzed using Comet Assay IV software. Comet slides were prepared, lysed, treated with RNase and proteinase K, and electrophoresed in a neutral buffer. (C) Comet images were captured using an epifluorescence microscope (Carl Zeiss), Scale bar, 50 μ m. (D) The tail moment of comets in (C) was analyzed using Comet Assay IV software. no, no CPT-treated worms.

DSBs were detected following treatment with IR or CPT, which is in agreement with studies using the neutral comet assay in human cells (Lankinen et al., 1996; Olive et al., 1990). Comet tails were barely detected under the neutral comet assay without treatments with ribonuclease A and proteinase K (unpublished data). The use of ribonuclease A and proteinase K in this study improved sensitivity of the neutral comet assay. Since DNA strand breaks detected by the glyoxal comet assay come from both single-strand breaks and double-strand breaks, greater tail moments would be expected than those from only double-strand breaks. However, the tail moment detected by the neutral comet assay was greater than that by the glyoxal comet assay. Because sample treatment and the electrophoresis conditions such as volts, currents, and times are different in two assays, it is hard to compare the tail moments directly. Nonetheless, these experiments provided evaluation of the comet assays as a reliable tool to study DNA strand breaks in *C. elegans*.

DNA damage levels, as measured by tail moment, remained high in *brc-1* nuclei at 24 h after IR, compared to 1 h. DSBs were increased after CPT treatment during recovery times in *brc-1* mutants. CPT-induced DNA strand breaks can be converted to DSBs by DNA replication in S-phase and the DSBs can be leveled off at a certain time if not repaired. These results suggest that DSB repair is defective in *brc-1*. Similarly, human cells deficient in *BRCA1* are more sensitive to ionizing radiation. DSB repair kinetics measured by the comet assay or pulsed

field gel electrophoresis in *BRCA1* mutants or *BRCA1* missense mutants showed that these mutants failed to repair DSBs efficiently (Chen et al., 2014; Scully et al., 1999).

When mammalian cells are exposed to IR, aggregates of repair proteins called nuclear foci appear within their nuclei (Bekker-Jensen et al., 2006). These foci contain proteins involved in HR including RAD51, RAD52, and RAD54, and form in response to DNA damage. Thus, it is thought that they represent sites at which recombinational repair reactions occur (Essers et al., 2002). RAD51 foci formation has served as an indicator of the induction of radiation-induced DSBs and DNA repair (Miyazaki et al., 2004; van Veelen et al., 2005). In the study of *C. elegans* DSB repair, DSBs induced by IR have been detected by RAD-51 foci in mitotic germline nuclei of *C. elegans* and DSBs formed during meiotic recombination have been also detected by RAD-51 foci (Adamo et al., 2008; Boulton et al., 2004; Garcia-Muse and Boulton, 2005).

We analyzed RAD-51 foci formation over time after exposure to IR. In wild-type N2, the percentage of germline nuclei showing RAD-51-positive foci decreased with increasing post-irradiation time (Fig. S1). In contrast, the decrease in foci in *brc-1* was less than that in N2, indicating less efficient DSB repair. Even though BRC-1 deficiency is associated with a DSB repair defect, *brc-1* mutants remain competent for RAD-51 foci formation after IR (Boulton et al., 2004), indicating that BRC-1 is dispensable for the recruitment of RAD-51 to sites of DSBs (Polanowska et al., 2006). Our results suggest that BRC-1 may

be involved in a step after RAD-51 binds to DSBs. In mammalian cells, BRCA1 aggregates into nuclear foci after exposure to IR and appears to associate with RAD51 (Powell and Kachnic, 2003). However, radiation-induced RAD51 foci still form in BRCA1-deficient tumor cells (Nakada et al., 2012; Scully et al., 1999).

IR-induced DSBs in mammalian cells have been also detected by immuno staining of γ -H2AX, which is a marker for DNA strand breaks (Paull et al., 2000). DSB repair kinetics measured by the comet assay correlated with γ -H2AX foci association. Quantitative studies further indicated that the extent of γ -H2AX formation is proportional to the number of DSBs and that their appearance and disappearance correlate very well with the kinetics of DSB repair (Balajee and Geard, 2004; Olive, 2011). Since antibodies against γ -H2AX in *C. elegans* are not available, we could not examine the formation of γ -H2AX foci in N2 or *brc-1* mutants.

In summary, we have shown that the dissected mitotic germline region can be used for the comet assay in *C. elegans* and that DNA strand breaks can be detected by glyoxal or neutral comet assays. These assays can be used for the detection of DNA strand breaks induced by other DNA damaging agents and for measuring DNA repair kinetics in *C. elegans*.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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