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Caenorhabditis elegans dna-2 is involved in DNA repair and is essential for germ-line development

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Abstract ***Caenorhabditis elegans*** **germ cell proliferation and development were severely damaged in second generation** ***dna-2*** **homozygotes. Even in the first generation, a much higher incidence of aberrant chromosomes in oocytes and resultantly higher embryonic lethality were found vs. wild type, when DNA breaks were induced by γ-rays or camptothecin. The deficiency of** ***dna-2*** **in combination with RNA interference on** ***mre-11*** **gene expression synergistically aggravated germ-line development, especially oocyte formation. These results suggest that** ***C. elegans*** **Dna-2 is involved in a DNA repair pathway paralleling homologous recombination or non-homologous end joining with** ***mre-11*** **participation.**

Key words: *dna-2*; Germ-line; DNA repair; *mre-11*; *Caenorhabditis elegans*

1. Introduction

Dna-2 is a highly conserved helicase-nuclease protein, first identified in *Saccharomyces cerevisiae* through screening for temperature-sensitive mutants defective in DNA replication in vitro [1–4]. Sequence analysis showed that Dna-2 in *S. cerevisiae* (ScDna2) has a RecB-type nuclease domain [5]. The endonuclease activity of Dna-2 in conjunction with Rad27/ScFen-1 is essential in Okazaki fragment processing [6] by ensuring the removal of both RNA primer and polymerase α-synthesized DNA [7]. ScDna2 also has a weak 5′→3′ DNA helicase activity in the C-terminus [8], which is not necessary for cell viability [9]. *Scdna2* mutants defective in the helicase or ATPase activity displayed sensitivity to methyl methanesulfonate, whereas those defective in endonuclease activity were not sensitive, suggesting that helicase activity plays a role in DNA repair [6]. Recently, it was reported that ScDna2 is required for telomerase-dependent telomere synthesis [10] and is needed for normal life span [11].

In our previous paper [12], we reported that all the essential motifs for nuclease and ATPase/helicase activities are conserved in the open reading frame (ORF) of *Caenorhabditis elegans* Dna-2 (Ce-Dna-2). A *C. elegans* mutant lacking 611 nucleotides in the transcribed region of the *dna-2* gene was screened out of a randomly mutated *C. elegans* pool. This deletion not only removed the ATPase/helicase domain at the C-terminal but also was predicted to abolish nuclease activity of Ce-Dna-2 in comparison with essential amino acid sequences in ScDna2. Homozygous *Ce-dna-2* mutants showed severe phenotypes such as embryonic lethality and reduced brood size, dependent on growth temperature [12]. In this study, we examined functions of Ce-Dna-2 in germ-line development, and also investigated its involvement in repair of DNA damage.

2. Materials and methods

2.1. *Germ-line phenotypes of* dna-2 C. elegans

C. elegans dna-2 mutant was isolated by mutant pool screening and backcrossed with strains N2 and *sqt-1*(*sc13*) alternately several times to remove possible mutations in other genomic regions [6]. The *dna-2* mutant with a cleared genomic background was crossed again with strain *sqt-1*(*e1350*) so that *dna-2* homozygotes could be visibly distinguished from wild type *dna-2*(+/+) and heterozygous *dna-2*(+/−) worms, which were dumpy and roller, respectively. Second generation homozygotes (*m*−*z*−) were obtained from first generation homozygous (*m*+*z*−) mutants that had been produced by a heterozygous mother. The oocytes and germ cells within *dna-2*(*m*+*z*−) and *dna-2*(*m*−*z*−) worms were observed microscopically with Nomarski optics (DMR HC, Leica). For nuclear staining, gonads were extruded by decapitating adult *C. elegans*, fixed in 3% formaldehyde and 0.1 M K_2HPO_4 (pH 7.2) for 1 h, and post-fixed with cold (−20°C) 100% methanol for 5 min. After treatment with 4′,6′-diamidino-2-phenylindole (DAPI), the specimen was observed under a fluorescence microscope (DMR HC, Leica).

2.2. *Effects of a prior temperature shift on the viability of* dna-2 *embryos*

In a temperature up-shift experiment, *dna-2*(*m*+*z*−) worms grown at 20°C for 3 days were transferred to new NGM plates pre-warmed to 25°C. *dna-2*(*m*+*z*−) worms were allowed to lay eggs for 2 h and then transferred to new NGM plates every 2 h. The viability of *dna-2*(*m*−*z*−) embryos to L1 larvae was counted 20 h later for each batch of embryos collected between transfers. In a temperature down-shift experiment, procedures were the same except the incubation temperature was reduced from 25°C to 20°C. As a 0 h control, *dna-2*(*m*−*z*−) embryos collected for 2 h at 20°C (or 25°C) were transferred to plates at 25°C (or 20°C) and their survival to L1 larvae was counted after 20 h.

2.3. *Sensitivity of* dna-2 *germ cells to DNA damages*

In order to examine the γ-ray sensitivity of meiotic germ cells, *dna-2*(*m*+*z*−) *C. elegans* worms at the young adult stage were irradiated

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Abbreviations: Ce-Dna-2, *Caenorhabditis elegans* Dna-2; dsRNA, double-stranded RNA; DAPI, 4′,6′-diamidino-2-phenylindole; ORF, open reading frame; RNAi, RNA interference

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by a ^{137}Cs source (IBL 437C, CIS Biointernational) at doses of 30, 60, and 120 Gy and then transferred to new plates after 6 h. Worms were allowed to lay eggs for 12 h and viability of the progeny to L1 larvae was measured 20 h later. To measure sensitivity to camptothecin (Sigma-Aldrich), young adult *dna-2*(*m*+*z*−) worms were grown on NGM plates containing 0.001, 0.01, 0.1, 0.2, 0.4, or 0.8 mM of camptothecin for 6 h and then transferred to drug-free plates, where embryos were collected for 12 h. Viability of their progeny was measured as described above. As controls, sensitivities to γ-rays and camptothecin were also measured for the wild type N2 strain. In order to examine oocyte chromosomes after γ-irradiation, gonads were dissected from wild type and *dna-2*(*m*+*z*−) worms 18 h later, stained with DAPI, and then observed with a fluorescence microscope.

2.4. *Bacteria-mediated RNA interference (RNAi) of* mre-11 *in* dna-2 *mutants*

The yk133b9 EST clone of a *C. elegans mre-11* homologue (ZC302.1) was obtained from Dr. Y. Kohara (National Institute of Genetics, Japan), digested with *Xba*I and *Xho*I enzymes, and inserted into vector pPD129.36(L4440) [13] containing two convergent T7 polymerase promoters in opposite orientations separated by a multicloning site. Plasmid DNA was transformed into *Escherichia coli* HT115(DE3) (W3110, *rnc14*::ΔTn*10*) cells using an electroporator (Invitrogen). Cells harboring plasmid DNA were directly applied onto agar plates composed of standard NGM/agar medium supplemented with 100 μg/ml ampicillin, 12.5 μg/ml tetracycline, and 0.4 mM isopropyl-β-D-thiogalactose, and then cultured overnight at

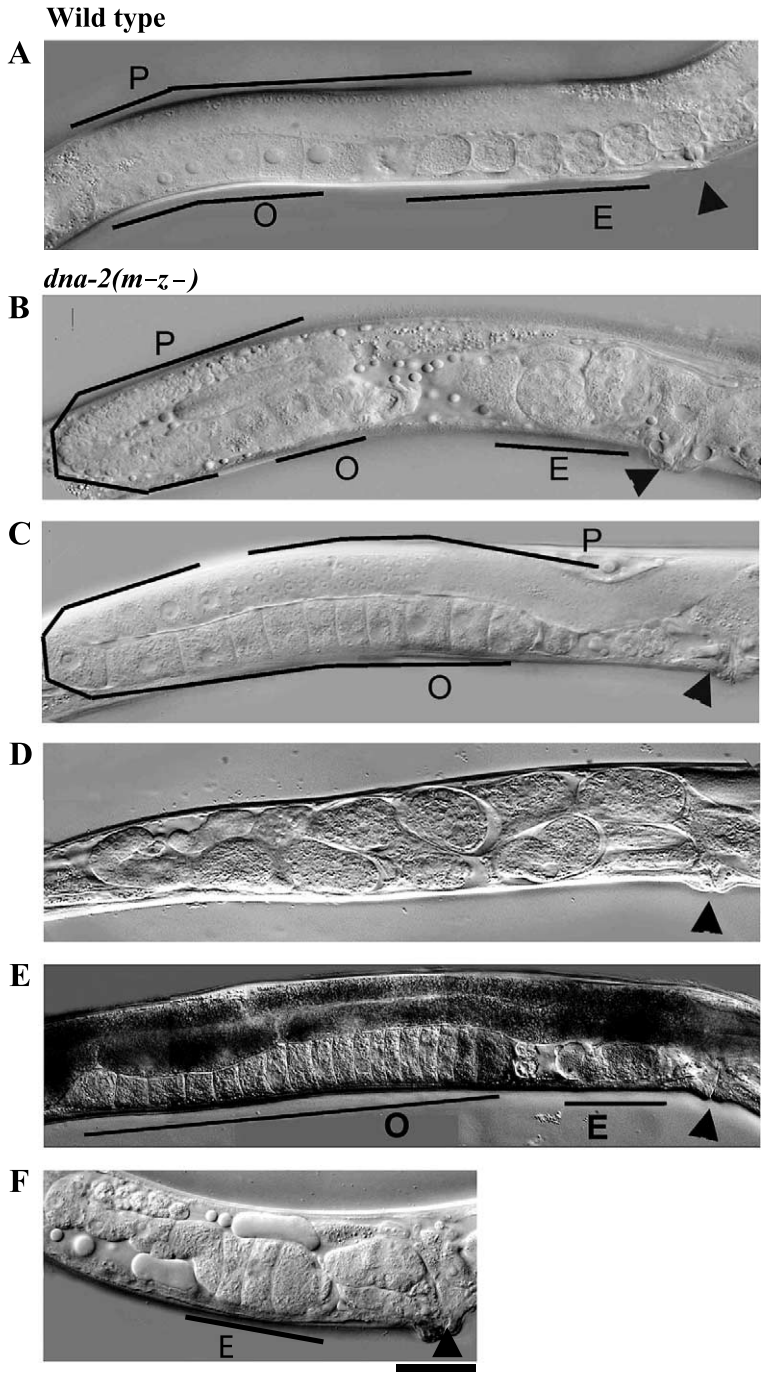

Fig. 1. Phenotypes of *C. elegans dna-2*(*m*−*z*−) gonads under Nomarski optics. A: Wild type. B: Expanded pachytene stage area and small number of oocytes. C: Distally expanded oocyte region. D: Gonad full of unlaid and arrested embryos. E: Excessive number of oocytes in proximal gonad. F: Small gonad. Arrowhead, vulva; P, pachytene stage; O, oocytes; E, embryos. Scale bar, 50 μm.

room temperature. On each plate covered with *E. coli* cells producing double-stranded (ds) RNA of *mre-11*, wild type N2 worms, heterozygous *dna-2* mutants, or homozygous *dna-2* mutants at the L4 stage were placed and grown for 24 h. Worms at the adult stage were allowed to lay eggs for 12 h, and hatching of F1 embryos was scored. Hatching was also scored for F2 embryos produced by F1 worms maintained on dsRNA feeding plates.

3. Results

3.1. Abnormal germ-line development in dna-2 mutants

A *dna-2* deletion mutant was isolated previously from a randomly mutated *C. elegans* pool, and reproduction phenotypes were examined [12]. The first generation homozygote designated *dna-2*(*m*+*z*−) showed mild phenotypes at 20°C, but *dna-2*(*m*−*z*−) worms of the next generation produced very small broods of embryos with ensuing high lethality [12]. In this study, we examined whether this phenotype was associated with any defects in germ-line development. The *dna-2*(*m*+*z*−) worms did not show any significant morphological abnormality in the gonads. However, various abnormalities appeared in gonads of *dna-2*(*m*−*z*−) worms (92 total adults, 4–8 days old from birth as embryos) such as pachytene stage cells expanded to the proximal gonad arm (23%, Fig. 1B), oocytes expanded to the distal gonad arm (9%, Fig. 1C), and excessive numbers of unlaid embryos in the uterus (20%, Fig. 1D). In addition, deformed oocytes (Fig. 1B, 40%), excessive oocytes compactly packed in the proximal gonad arm (Fig. 1E, 8%), small gonads (Fig. 1F, 34%), and abnormal migration of the distal gonad tip (8%, Fig. 1B) were observed.

To examine germ-line nuclei in *dna-2*(*m*−*z*−) worms more closely, gonads were dissected out and stained with DAPI as shown in Fig. 2. Some mutant gonads contained an expanded pachytene area and a smaller number of oocytes (Fig. 2B), which was very similar to the phenotype in Fig. 1B. Other mutant gonads showed an expanded region of oocytes (Fig. 2C) with oocytes undergoing endomitosis (27% among 100 gonads), which was also shown in Fig. 1C, and a great reduction in size (Fig. 2D). When wild type oocytes were further magnified, six condensed bivalent chromosomes prior to meiosis I were observed as shown in Fig. 2E (left panel). In contrast, chromosomes in the oocytes of *dna-2*(*m*−*z*−) worms were aberrant in both number and size of chromosomes (Fig. 2E, right panel). This aberrancy in oocyte chromosomes was likely a primary cause of both severe embryonic lethality and a 10 times higher male frequency (data not shown) among *dna-2*(*m*−*z*−) progeny than in wild type worms.

3.2. Relative importance of Dna-2 during embryogenesis and germ-line development

The hatching rate of *dna-2*(*m*−*z*−) embryos laid by *dna-2*(*m*+*z*−) worms was previously measured to be 90% at 20°C and 9% at 25°C [12]. By taking advantage of this temperature dependence of embryonic lethality, the relative importance of Dna-2 during embryogenesis and germ cell development was evaluated. When the incubation temperature of

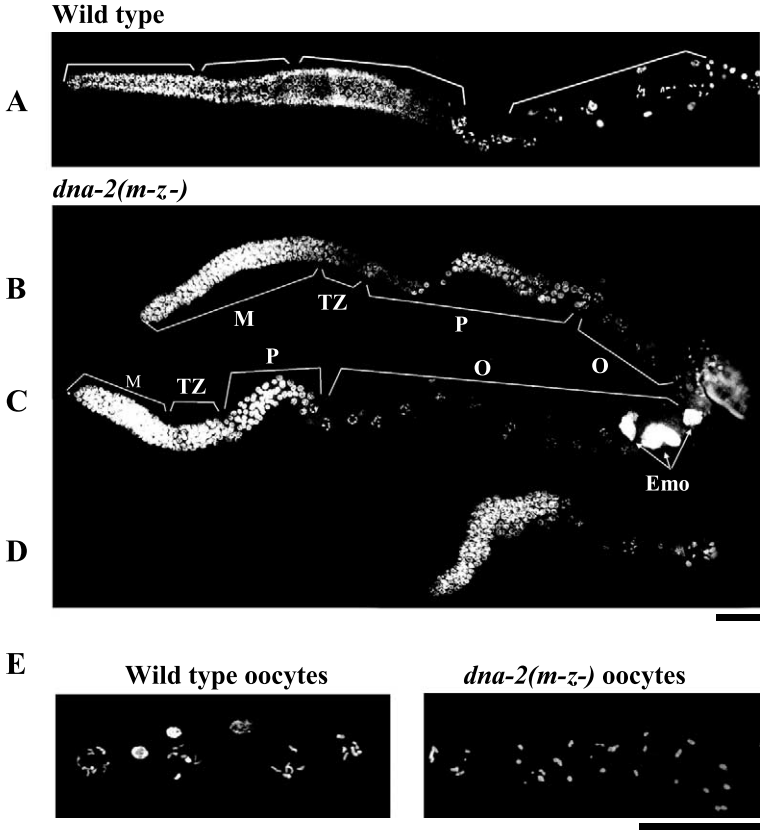

Fig. 2. Nuclear staining of gonads in *dna-2*(*m*−*z*−) worms. Gonads were dissected from wild type and *dna-2*(*m*−*z*−) worms, and stained with DAPI. A: Wild type. B: Expanded pachytene region and reduced number of oocytes. C: Expanded oocyte region, stunted pachytene region, and endomitotic oocytes. D: An undersized gonad. M, mitotic region; TZ, transition zone; P, pachytene stage; O, oocytes; Emo, endomitosis. E: Condensed chromosomes in oocytes of wild type and *dna-2*(*m*−*z*−) adult worms. Scale bars, 50 μm.

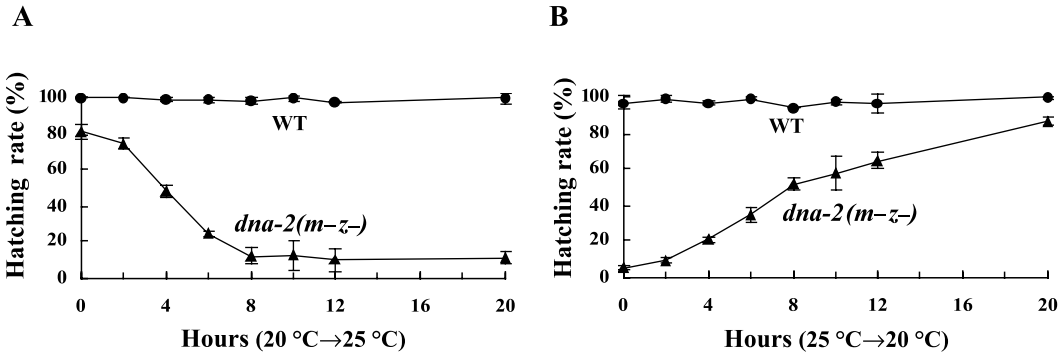

Fig. 3. Effects of temperature shift at various stages of germ cell development and embryogenesis on survival of *dna-2*(*m*−*z*−) embryos. A: Wild type (WT) or *dna-2*(*m*+*z*−) worms were transferred to new NGM plates every 2 h after the shift from 20°C to 25°C to collect embryos laid during 2 h intervals. Hatching rate was scored 20 h later. (B) Experimental procedure same as A except the temperature shift was reversed, from 25°C to 20°C. Over 100 embryos were tested for each data point of a single experiment set, and each experiment was repeated three times. Error bars indicate S.D.

dna-2(*m*+*z*−) worms was up-shifted from 20°C to 25°C, the hatching rate of *dna-2*(*m*−*z*−) embryos dropped sharply to 10% within 8 h after the temperature shift and remained at that level until 20 h (Fig. 3A). In contrast to this rapid decline during 2–8 h, relatively mild reductions of hatching rate to 83% and 78% from the corresponding value of 90% at 20°C [12] were obtained for the embryos collected at 0 and 2 h, respectively. This result suggests a less critical role of Dna-2 during embryogenesis than during prior germ-line development. When the incubation temperature was down-shifted from 25°C to 20°C, the hatching rate increased sharply during 2–8 h, again indicating the important function of Dna-2 during the late pachytene stage and oocyte development (Fig. 3B). In addition, the survival rate gradually increased until 20 h, suggesting the action of Dna-2 from the early pachytene stage.

3.3. dna-2 *meiotic germ cells are hypersensitive to DNA breaks*

Since mitotic proliferation in *dna-2*(*m*+*z*−) gonads was apparently normal, but not in *dna-2*(*m*−*z*−) gonads, *dna-2*(*m*+*z*−) worms were used in order to investigate the role of *C. elegans* Dna-2 protein during meiosis. Sensitivity to DNA damage was measured after pre-irradiating *dna-2*(*m*+*z*−) worms with γ-rays and then counting the hatching of *dna-2*(*m*-*z*−) embryos derived from pachytene stage germ cells. The hatching rate of embryos laid during 6–18 h after γ-ray irradiation at doses of 30 and 60 Gy was about 41% and 29%, respectively, which was significantly lower than the corresponding values (30 Gy, 79%; 60 Gy, 52%) in the wild type (Fig. 4A). In wild type oocytes derived from germ cells irradiated at the pachytene stage with 30, 60, and 120 Gy of γ-rays, chromosomes appeared normal with six bivalents. In contrast, univalent chromosomal copies increased in oocytes of *dna-2*(*m*+*z*−) worms, slightly at 60 Gy and greatly at 120 Gy, where chromosomal fragmentation was also observed (Fig. 5).

Beside ionizing radiation, camptothecin was used to induce DNA breaks and sensitivity of the germ cells in *dna-2*(*m*+*z*−) worms was measured the same way. Embryos produced from germ cells exposed to camptothecin at the pachytene stage showed much higher sensitivity to camptothecin than wild type embryos (Fig. 4B). This suggests that Dna-2 protein participates in the repair of single- or double-stranded DNA breaks resulting from the tertiary cleavable complex of camptothecin, DNA topoisomerase I, and DNA [14].

3.4. *Double deficiency of* dna-2 *and* mre-11 *synergistically increased germ-line abnormalities and embryonic lethality in* C. elegans

In order to determine any relationship of Dna-2 with Mre-11 in dsDNA break repair, *mre-11* gene expression was inhibited by bacteria-mediated RNAi in *dna-2* mutants. Mre-11

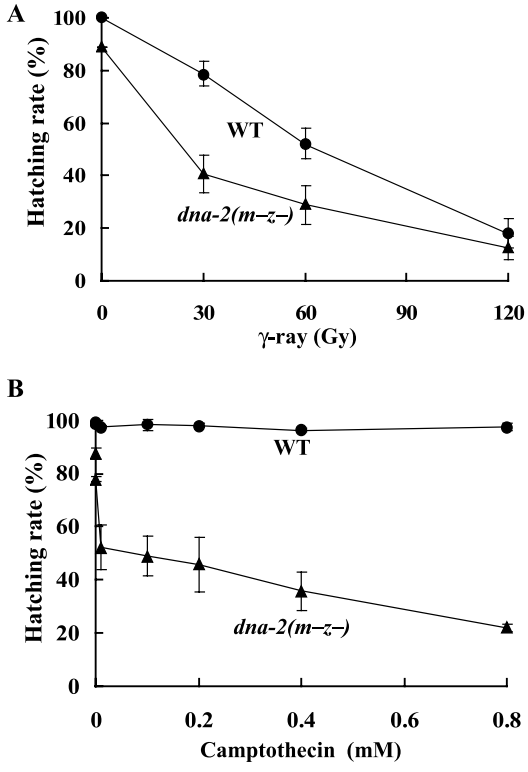

Fig. 4. Hypersensitivity of *dna-2* mutants to DNA damage. Pachytene stage germ cells in wild type (WT) and *dna-2*(*m*+*z*−) worms were treated with γ-rays or camptothecin, and hatching of the embryos (*dna-2*(*m*−*z*−) derived from the germ cells) was scored. Over 100 embryos were tested for each point of a single experiment set, and each experiment was repeated three times. Error bars indicate S.D.

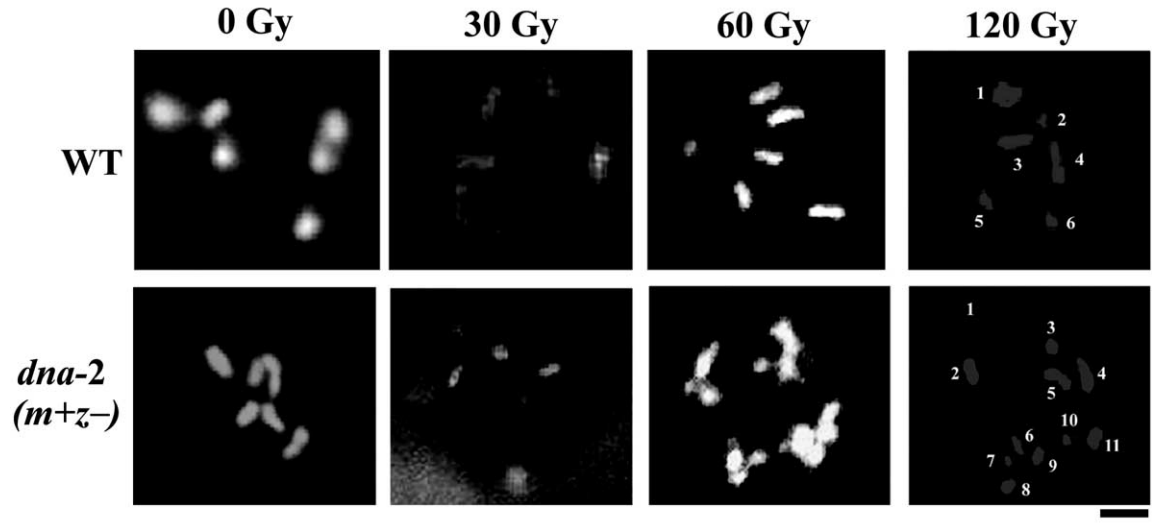

Fig. 5. Chromosomal aberrations in *dna-2* oocytes induced by γ-irradiation. Germ cells in wild type (WT) and *dna-2*(*m*+*z*−) worms at the young adult stage were irradiated with γ-rays at various doses. The gonads were extruded out of the worms 18 h later and stained with DAPI. Oocytes at the diakinesis stage were likely to be at the pachytene stage at the time of irradiation. Chromosomes in the oocytes of *dna-2*(*m*+*z*−) worms appeared intact at 30 Gy, were clumped at 60 Gy, and showed an increased number, probably due to univalence, and fragmentation at 120 Gy. Scale bar, 2 μm.

exo/endonuclease, as a tertiary complex (M/R/N) with Rad50 and NBS1, is involved in repair of dsDNA breaks mediated by either homologous recombination or non-homologous end joining pathways. Beside the role in DNA repair, the M/R/N complex regulates cell cycle checkpoint in response to DNA breaks [15,16]. When a wild type N2 strain was fed with *E. coli* cells expressing *mre-11* dsRNA, F1 embryos showed no effects of the RNAi, and F2 embryos showed only 8% lethality (Table 1). Likewise, F1 homozygous embryos of the genotype *dna-2*(*m*+*z*−) were unaffected by RNAi. This suggests that *mre-11* gene expression was not effectively inhibited, based on the report that *mre-11 C. elegans* mutants showed 98% embryonic lethality [17]. In spite of this incomplete inhibition of *mre-11* expression, F2 homozygous embryos showed 71% lethality when RNAi was performed from the L4 stage of heterozygotes throughout the F1 heterozygote generation. The lethality of F2 embryos of the genotype *dna-2*(*m*+*z*−) was much higher than the corresponding value of 8% for the F2 embryos of the wild type strain, suggesting a synergistic effect of the double deficiency of *dna-2* and *mre-11*. F1 embryos of the genotype *dna-2*(*m*−*z*−) showed 95% lethality upon RNAi of *mre-11*, which was a marked increase compared with the corresponding value of 10% for the homozygotes without RNAi, again showing synergism between *dna-2* and *mre-11* double deficiency. Reduced brood sizes (data not shown) accompanied embryonic lethality shown in Table 1, implicating abnormalities in germ-line development. Indeed, a major fraction of gonads in the first generation homozygotes fed with dsRNA contained a greatly reduced number of germ cells and almost no oocytes (Fig. 6). Although double deficiency of *dna-2* and *mre-11* affected germ cell development from the distal gonad arm, the most profound effect was no oocyte formation, which implied more critical roles of the two genes at pachytene or diplotene stages than at the mitotic germ cell stage.

Table 1
Embryonic lethality of *dna-2* mutants upon RNAi of *mre-11*

Embryo genotype	Without RNAi	*mre-11* RNAi	
		F1[a]	F2[a] embryos
Wild type	0 (<1)	0 (<1)	8 (±1)
dna-2(*m*+*z*−)	0 (<1)	0 (<1)	71 (±2)
dna-2(*m*−*z*−)	10 (±2)	95 (<1)	100 (<1)

[a]Wild type, heterozygous *dna-2*(+/−), or first generation homozygous *dna-2*(*m*+*z*−) worms at the L4 stage were placed on an *E. coli* lawn producing *mre-11* dsRNA. Lethality was scored for F1 embryos of the indicated genotypes and also for F2 embryos. Each number was obtained by scoring more than 150 embryos with three repetitions.

4. Discussion

In our previous paper, *C. elegans* Dna-2 protein was highly expressed in meiotic germ cells and oocytes as well as in mitotic germ cells [12]. The localization of *C. elegans* Dna-2 in mitotic germ cells was expected, because the protein is presumably involved in DNA replication, as in yeast. However, the strong expression of Ce-Dna-2 protein in meiotic germ cells and oocytes suggested that the protein is involved in a process other than DNA replication. Although the role of ScDna2 in DNA replication is well established, little is known about its possible role in meiotic cells. In order to investigate meiotic functions of Dna-2, a *dna-2 C. elegans* mutant with a premature termination after amino acid 378 in the ORF of 1069 amino acids was examined as to the germ-line in this study [12]. In the gonad of *dna-2*(*m*+*z*−) worms, the progeny of a heterozygous mother, no distinct phenotypes were observed, suggesting that maternal Dna-2 was sufficient for germ cell development. In the gonad of *dna2*(*m*−*z*−) hermaphrodites of the next generation, apparent phenotypes did not appear until the L4 stage, suggesting that Dna-2 was not required for gonadogenesis. Once the young adult gonad began to produce oocytes, abnormal phenotypes appeared gradually in the pachytene stage germ cells and oocytes (Figs. 1 and 2). The expanded pachytene region (Figs. 1B and 2B) could be due to a defective exit from the stage, which was

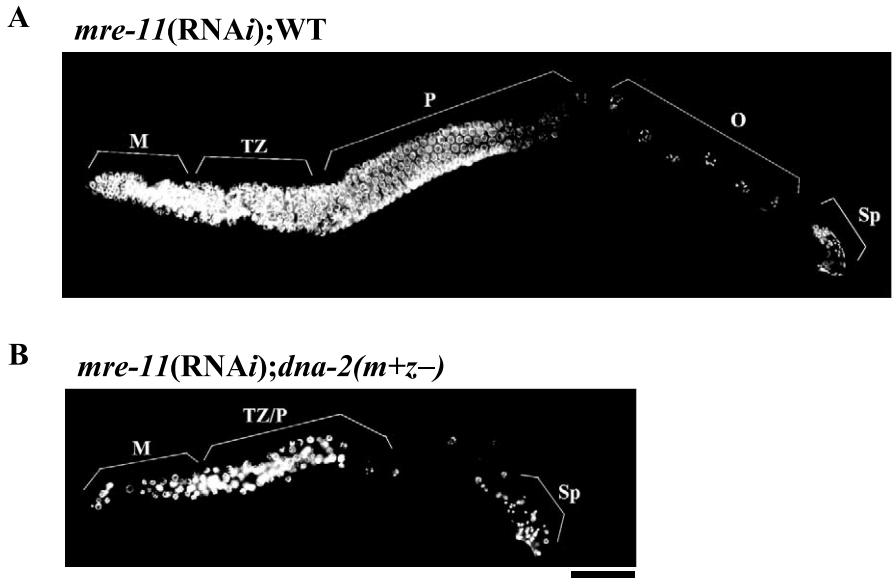

Fig. 6. Double deficiency phenotypes of *dna-2* and *mre-11* in *C. elegans* gonads. RNAi of *mre-11* was performed in wild type (WT) and *dna-2*(*m*+*z*−) mutants and their gonads were separated from the worms to be stained with DAPI. A: RNAi of *mre-11* in a wild type gonad. B: RNAi of *mre-11* in a *dna-2*(*m*+*z*−) gonad. M, mitotic region; TZ, transition zone; P, pachytene stage; O, oocytes; Sp, spermatheca. Scale bar, 50 μm.

also induced by the ablation of somatic gonad cells such as sheath and spermathecal cells [18]. This conjecture agrees with the transient expression of green fluorescent protein-fused Dna-2 in the spermathecae and uterus at the L4 stage [12]. The distally expanded oocyte region in Figs. 1C and 2C and the excessive number of compactly packed oocytes (Fig. 1E) in the proximal gonad arm were likely to have resulted from abnormalities in oocyte maturation, ovulation, or fertilization. As *dna-2*(*m*−*z*−) adults aged, the frequency of shortened and severely deformed gonad arms increased further (Fig. 2D). As shown in the DAPI-stained nuclei of Fig. 2E, the number and size of chromosomes in oocytes of *dna-2*(*m*−*z*−) worms were aberrant compared with those of wild type oocytes. Chromosomal mis-segregation subsequent to abnormal pairing of homologous chromosomes probably resulted in the 10-fold increase in male production from *Ce-dna-2*(*m*−*z*−) worms.

Helicase activity of ScDna2 might be particularly important for the methyl methanesulfonate-induced DNA repair process [9]. In order to examine whether *C. elegans* Dna-2 is involved in DNA repair, we measured the sensitivity of *dna-2* mutants to DNA damage. As shown in Fig. 4, DNA strand breaks induced by γ-rays and camptothecin caused much more severe embryonic lethality in the germ cells of *dna-2*(*m*+*z*−) worms than in wild type, suggesting that Dna-2 plays an important role in repair of dsDNA breaks. This argument is further supported by aberrant chromosomes induced by ionizing radiation in the oocytes of *dna-2*(*m*+*z*−) worms, as shown in Fig. 5.

The relative importance of Dna-2 during embryogenesis and meiotic germ cell development was examined by carrying out temperature shift experiments on *dna-2*(*m*+*z*−) adult worms (Fig. 3). Although a slight decrease in hatching rate of embryos collected during the first 2 h after temperature upshift supports a role of Dna-2 during embryogenesis, its more critical action during meiotic germ cell development is implied by the more rapid drop of the hatching rate during the 2–8 h period. Results from the temperature down-shift experiment suggest the action of Dna-2 from early pachytene stage onwards. Dna-2 is probably involved in the repair of spontaneous DNA damage during homologous recombination of meiotic prophase. In accord with this argument, synergistic effects of double deficiency of *dna-2* and *mre-11* appeared as a severely under-developed germ-line with respect to cell proliferation and differentiation, especially as a failure to form any oocytes (Fig. 6). Synergism between the two genes suggests the possibility that *dna-2* may act in a DNA repair pathway parallel to homologous recombination or non-homologous end joining involving *mre-11*.

In summary, we have shown in this report that Dna-2 has distinct roles in mitotic and meiotic cells during *C. elegans* development. Dna-2 in mitotic cells has been shown to be required for embryogenesis [12] and mitotic germ cell proliferation, and Dna2 in meiotic cells is shown here to be required for repair of induced and also probably of spontaneous DNA damage. The next step will be to determine in which part of DNA repair or possibly of meiotic recombination Dna-2 is involved.

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