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Genetic and cytological characterization of the recombination protein RAD-51 in *Caenorhabditis elegans*

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Abstract We investigated the role of *Caenorhabditis elegans rad-51* during meiotic prophase. We showed that *rad-51* mutant worms are viable, have no defects in meiotic homology recognition and synapsis but exhibit abnormal chromosomal morphology and univalent formation at diakinesis. During meiosis RAD-51 becomes localized to distinct foci in nuclei of the transition zone of the gonad and is most abundant in nuclei at late zygotene/early pachytene. Foci then gradually disappear from chromosomes and no foci are observed in late pachytene. RAD-51 localization requires the recombination genes *spo-11* and *mre-11* as well as *chk-2*, which is necessary for homology recognition and presynaptic alignment. Mutational analysis with synapsis- and recombination-defective strains, as well as the analysis of strains bearing heterozygous translocation chromosomes, suggests that presynaptic alignment may be required for RAD-51 focus formation, whereas homologous synaptonemal complex formation is required to remove RAD-51 foci.

Introduction

Meiosis is a specialized cell division that leads to the reduction of the diploid chromosome complement and results in the production of haploid gametes. Compared with mitotic division, the first meiotic division is unique as homologous parental chromosomes associate with each other to enable the reciprocal exchange of corresponding portions and the orderly disjunction of chromosomes.

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During meiotic recombination, double-stranded DNA breaks (DSBs) are deliberately generated by the type II topoisomerase-like Spo11p nuclease in order to trigger the exchange of sequences between chromosomes (Bergerat et al. 1997; Keeney et al. 1997; Dernburg et al. 1998; Keeney et al. 1999; Cervantes et al. 2000; Mahadevaiah et al. 2001). In addition to *spo-11*, studies in budding yeast also implicated further genes including the *mre-11* nuclease, which is involved both in DSB generation and processing. Meiotic DSBs are repaired by recombinational DNA repair, which may result in reciprocal recombination (=crossing over). Crossing over produces chiasmata that, in combination with sister chromatid cohesion, allow the formation of stable bivalents. Moreover, crossing over contributes to the genetic variability that underlies evolution.

One of the key enzymatic activities needed for recombinational repair is DNA strand exchange conferred by Rad51p, a homolog of the bacterial RecA protein (Shinohara et al. 1992; Paques and Haber 1999). To allow for strand exchange, DSBs are resected such that extended single-stranded 3' overhangs are produced. These single-stranded DNA tracks are loaded with Rad51p and form a nucleoprotein filament (Ogawa et al. 1993) in which single-stranded DNA assumes a stretched configuration that makes it competent to search for and invade a homologous double-stranded DNA sequence to initiate strand exchange. As a consequence of recombinational repair, genetic information is transferred from the template molecule to the repaired DNA molecule.

Meiotic recombination requires the recognition and pairing of homologous chromosomes. In meiotic prophase, two axes representing the two homologous chromosomes start to align at a distance (presynaptic alignment) before they associate intimately via the synaptonemal complex (SC) (for reviews see Loidl 1990; Roeder 1997; Zickler and Kleckner 1999). In the budding yeast, mouse and *Arabidopsis*, meiotic DSBs were found to precede and to be required for chromosome synapsis (Romanienko and Camerini-Otero 2000; Grelon et al. 2001; Hunter and Kleckner 2001; Mahadevaiah et

al. 2001). The sites of initial chromosome association are thought to be sites where DSBs are generated. Based on these cytological data it was thus postulated that a RAD-51 nucleofilament invades a complementary DNA sequence on the homologous chromosome, leading to subsequent synapsis. In *Caenorhabditis elegans*, however, DSBs are not a precondition for SC formation (Dernburg et al. 1998).

Several features of the nematode worm *C. elegans* make it a particularly useful model to study the mechanisms underlying DNA damage response, meiotic recombination and meiotic chromosome pairing. Within the gonad cells are organized in a highly polarized way with the most distal end of the ovotestes containing a mitotic stem cell compartment that is followed by cells in different stages of meiotic prophase. Two states of meiotic chromosome pairing, namely synapsis-independent association (which we refer to as presynaptic alignment throughout the text) and synapsis can be separated by mutational analysis (MacQueen et al. 2002), allowing the assignment of biochemical and genetic events of recombination and pairing to processes at the cytological level by immunolocalization of the respective proteins. In addition, *C. elegans* is a multicellular system that circumvents the intrinsic complication of mammalian systems where deletion of genes involved in meiotic recombination or chromosome pairing results in early embryonic lethality or tends to elicit programmed cell death, which confounds detailed cytological analysis in the respective mutants (Lim and Hasty 1996; Tsuzuki et al. 1996; Xiao and Weaver 1997; Luo et al. 1999; Yamaguchi-Iwai et al. 1999; Hirao et al. 2000).

The *C. elegans* genome project revealed that *C. elegans* has two *RecA* homologs, one of which, Y43C5A.6, is closely related to *rad-51* and termed both *Ce-rad-51* and *Ce-rdh1* (Rinaldo et al. 1998; Takanami et al. 1998). The other *RecA* homolog (C30A5.2), the inactivation of which by RNA interference (RNAi) or by transgene cosuppression did not show a distinct phenotype, is related to mammalian *rad-51C* and was shown to interact with RAD-51 in a two-hybrid assay (Boulton et al. 2002; Anton Gartner, unpublished). Initial, RNAi-based studies on *Ce-rad-51* suggested that RNAi depletion resulted in low viability of the offspring of worms, in bivalents that appeared not properly condensed, in a meiotic chromosome segregation defect, and in the activation of the DNA damage checkpoint pathway (Gartner and Hengartner 1998; Rinaldo et al. 1998; Takanami et al. 1998; Rinaldo et al. 2002). However, the cytological localization of meiotic recombination enzymes like RAD-51 has not been described in *C. elegans*. To further our understanding of meiosis-associated roles of *rad-51* and their correlation with meiotic chromosome pairing we embarked on a genetic and cytological analysis of the *C. elegans rad-51* ortholog.

Materials and methods

Worm strains

Strains of *C. elegans* were cultured as described by Brenner (1974). The wild-type strain background was Bristol N2, and the following mutations and chromosome rearrangements were used: the *rad-51* deletion *lg08701* (nucleotides 15810 to 14871 of Y43C5A.6) was isolated from a *C. elegans* deletion library and kindly provided by Elegene (Munich, Germany). *rad-51(lg08701)* was back-crossed five times with wild-type Bristol N2 and marked with *dpy13(e184)* and balanced by *nT1*. Balancer strains containing *eT1(III;V)* (BC2200, *dpy-18(e364)/eT1 III*; *unc-46(e177)/eT1 V*) and *hT1(I;V)* (KR1037, *unc-13(e51)/hT1*; *dpy-11(e224)/hT1[unc-42(e270)]V*) were provided by the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, Minn.). AV112, *mre-11(ok179) IV/nT1[unc-?(n754) let-?](IV;V)*, DR787, *dpy-13(e184) ama-1(m118) let-276(m240)/nT1 IV*; *+nT1 V* and MT5734, *nDf41 IV/nT1[unc-?(n754) let-?](IV;V)* were also obtained from the Caenorhabditis Genetics Center. AV115, *msh-5(me23)* (Kelly et al. 2000), AV106, *spo-11(ok79)* (Dernburg et al. 1998), and AV146, *chk-2(me64)* (MacQueen and Villeneuve 2001) strains were kindly provided by A.M. Villeneuve.

Production of antibodies

A polymerase chain reaction (PCR) fragment encoding the N-terminal 103 amino acids of *C. elegans* RAD-51 was cloned in frame as a glutathione S-transferase (GST) fusion into pGEX-4T-1 (Amersham, Uppsala, Sweden) and as a maltose binding protein (MBP) fusion into pMal-c2 (New England BioLabs, Beverly, Mass.). Recombinant GST and MBP fusions were expressed in *Escherichia coli* BL21 CodonPlus (Stratagene, La Jolla, Calif.), purified under native conditions by affinity chromatography using Glutathione-Sepharose 4B (Amersham, Uppsala, Sweden) and by amylose-resin affinity chromatography (New England BioLabs, Beverly, Mass.). The purified GST protein fusion was dialyzed against phosphate-buffered saline (PBS) and used for immunization of rabbits (Eurogentech, Seraing, Belgium). For anti-RAD-51 antibody affinity purification MBP-RAD-51 fusion protein was used. The MBP fusion was dialyzed against 1xPBS and 2 mg of total protein was covalently bound to an Affi-Gel 15 matrix (BioRad, Hercules, Calif.). Crude serum was diluted 1:1 in 20 mM TRIS, pH 7.5 and pre-cleaned by centrifugation and applied to the column to allow for the binding of the antibody. After washing with 10 mM TRIS-HCl, pH 7.5 and 10 mM TRIS-HCl, pH 7.5, 0.5 mM NaCl, antibodies were eluted with 100 mM glycine, pH 2.5 and the eluates were fractionated and immediately neutralized with 1.5 M TRIS, pH 8.0.

Gene knockdown

The RAD-51, HIM-3 and SYP-1 proteins were depleted by double-stranded RNA interference (RNAi). RNAi-mediated depletion of *rad-51* was performed as described in Gartner et al. (2000) by dsRNA injection (Fire et al. 1998). For *him-3* RNAi a PCR fragment was amplified out of cDNA of N2 worms using the primers 5'-TAATACGACTCACTATAGGGGCGGCCG-CG-GCGACGAAAGAGCAGATTG -3' and 5'-TAATACGACTCAC-TATAGGGGCGGCCGCTCTTCTTCGTAATGCCCTGAC-3', which contain the targeted sequence flanked by the T7 promoter (underlined sequence). For SYP-1 (F26D2.2) RNAi by injection, dsRNA was produced using the primers given by MacQueen et al. (2002). The dsRNA was prepared using a Promega (Madison, Wis.) in vitro transcription kit. The dsRNA was delivered by injection (~200 µg/ml) into gonads or intestines of young adults as described in Fire et al. (1998).

For REC-8 depletion a cosuppression line was constructed according to the schedule of Dernburg et al. (2000). In short, a 3.4 kb PCR product comprising the W02A2.6 sequence was

amplified from genomic DNA of N2 worms using the following primers: 5'-TCTTGAATAGGCTCCTGGGGTGCT-3' and 5'-ACCGTGCGGCACGAATCGTTTCAT-3'. This fragment was gel-purified and co-injected with the *rol-6(su1006)* marker. Offspring showing the roller phenotype were selected and lines with the *rec-8* deficiency phenotype were established. The lines used for further experiments were checked each time by 4',6-diamidino-2-phenylindole (DAPI) staining for the preservation of the phenotype.

Cytological preparation

Gonads were removed from hermaphrodites and transferred to a drop of M9 buffer (0.3% KH₂PO₄, 0.6% Na₂HPO₄, 0.5% NaCl, 1 mM MgSO₄) on a poly-L-lysine-coated microscope slide. An equal volume of 7.4% formaldehyde was added, and the material was gently squashed under a coverslip. Coverslips were removed by the dry ice method of Conger and Fairchild (1953), and the slides were transferred to 96% ethanol at -20°C for 5 min and then three times to 1×PBS at room temperature for 5 min each. Slides were then dehydrated in an ethanol series (40%, 70%, 96%, 2 min each), air dried and stored in the refrigerator until used for conventional chromosome staining or fluorescence in situ hybridization (FISH). For subsequent immunostaining, worms were prepared as above and squashed under a coverslip without formaldehyde fixation. After removal of coverslips, preparations were fixed in a series of methanol, methanol:acetone (1:1), and acetone for 5 min each at -20°C, and immediately transferred to 1×PBS without drying.

Immunostaining

Immunostaining was performed according to the standard protocol described in Pasierbek et al. (2001). Briefly, preparations were washed three times for 5 min in 1×PBS and blocked with 3% BSA in 1×PBS for 30 min at 37°C in a humid chamber. The primary antibody was applied and the specimen was incubated overnight at 4°C in a humid chamber. Antibodies were diluted in 1×PBS containing 3% BSA as follows: 1:100 anti-REC-8, 1:50 anti-RAD-51. After washing three times in 1×PBS, 0.1% Tween 20 secondary antibodies were applied in the following dilutions: anti-rabbit Cy3 (1:250), anti-rabbit-fluorescein isothiocyanate (FITC) (1:500) or anti-rat-FITC (1:500). After 30 min incubation at room temperature, slides were washed and mounted in Vectashield anti-fading medium (Vector Laboratories, Burlingame, Calif.) containing DAPI (2 µg/ml).

Fluorescence in situ hybridization

Cosmid F56C11 (~42 kb) from near the left end of chromosome I (obtained from Alan Coulson, Nematode Functional Genomics Group, Sanger Centre) was used as a FISH probe. The cosmid was labeled with digoxigenin-11-dUTP using the BioNick Labeling System (Life Technologies, Rockville, Md.) according to the instructions of the manufacturer. Labeled probe DNA was denatured and hybridized to denatured target sequences on cytological slides following the protocol described in detail by Pasierbek et al. (2001). Hybridized digoxigenin-labeled probes were detected with rhodamine-conjugated anti-digoxigenin, which generates red-fluorescing signals upon excitation by short wavelengths. Slides were mounted in Vectashield containing 2 µg/ml DAPI.

Cytological studies were performed with a Zeiss Axioskop epifluorescence microscope. For the quantification of RAD-51 immunofluorescence signals, images were taken using a DeltaVision setup (Applied Precision LLC, Issaquah, Wash.). Immunostained gonads were serially scanned with a scan depth of 0.2 µm using a 100× objective to generate a three-dimensional image. Stacks of images (50–60) were deconvoluted with up to ten iterations with the softWoRx software (Applied Precision LLC, Issaquah, Wash.) and finally projected to generate a single image. To allow a comparable quantification of the RAD-51 foci on different immunological samples, we mathematically subtracted the background (background intensity was defined as less than 5% of the maximal intensity of the RAD-51 foci), resulting in an image with distinct foci. Images were processed using Adobe Photoshop (Adobe Systems, Mountain View, Calif.).

Results

Isolation of a *rad-51* mutant

To study the role of *C. elegans* RAD-51 in meiosis we isolated a deletion mutant of the *rad-51* gene using a *C. elegans* deletion library. One 939 bp deletion was recovered (*lg08701*) that ablates the last three exons of *rad-51* and includes conserved catalytic domains needed for ATP hydrolysis and DNA strand exchange (Ogawa et al. 1993; Story et al. 1993) (Fig. 1A). We therefore

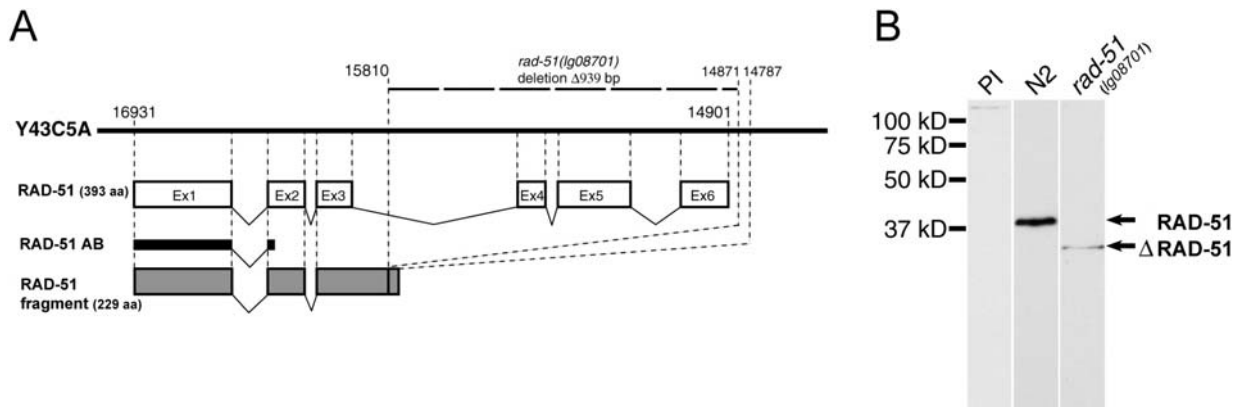


Fig. 1 A Map of the sequence region Y43C5A.6, which encodes RAD-51, with six exons. Also shown are the truncated protein of the *rad-51(lg08701)* allele and the region against which the antibody (AB) was raised. The mutation *rad-51(lg08701)* is a 939 bp deletion of the last three exons of *rad-51*, which contain the conserved domain for ATP-dependent strand exchange activity. B Immunoblot of *Caenorhabditis elegans* protein extracts. Affinity-

purified RAD-51 antibody was used for immunoblot analysis on total worm lysates (protein equivalent of ~50 worms loaded) of wild-type (N2) and *rad-51(lg08701)*. With preimmune serum (PI) no band is detected. The antibody recognizes a band of M_r 39,000 corresponding to RAD-51 in wild-type worms (N2) and the truncated protein in the mutant

consider this mutation to be a null allele of *rad-51*. The deletion strain was backcrossed five times to remove secondary mutations and the deletion was linked to *dpy-13*. Furthermore, to analyze the localization of RAD-51, a polyclonal antiserum against the first 103 amino acids of RAD-51 was generated and affinity purified. By immunoblotting, this antibody recognized a distinct band that migrates at a molecular weight of ca. M_r 39,000 and corresponds to the predicted molecular weight of RAD-51. *rad-51(lg08701)* worms showed a band of reduced intensity and a size that corresponds to the N-terminal RAD-51 truncation generated by the deletion (Fig. 1B).

Phenotypic analysis of the mutant

We next investigated the biological consequences of the *rad-51* deletion. As is the case with *rad-51(RNAi)* (Rinaldo et al. 1998, 2002; Takanami et al. 1998), the deletion of *rad-51* allows the survival of worms. While in *rad-51(RNAi)* animals up to ca 95% embryonic lethality and a high incidence of males had been found, we observed 100% lethality of embryos that were laid by *rad-51(lg08701)* worms. Of 1845 eggs laid by 32 different worms, no live embryos hatched after 2 days at 20°C, whereas in the *dpy13* control 370 embryos hatched from 376 eggs laid by 8 worms (1.6% lethality) under the same conditions. The complete lethality is likely due to the stronger phenotype of the genuine gene disruption as compared with the RNAi phenotype (Takanami et al. 1998). *rad-51(lg08701)* worms developed normally and at a similar pace to wild-type controls, suggesting that there is no major effect of *rad-51* on embryonic or larval cell cycle progression (data not shown).

The *rad-51(lg08701)* disruption led to elevated germ cell apoptosis that was further enhanced by irradiation (Fig. 2). This enhanced level of apoptosis is due to the activation of the *C. elegans* DNA damage checkpoint response (most likely due to unrepaired meiotic DSBs) and does not affect DNA damage-independent “physiological germ cell death” (Gumienny et al. 1999; Gartner et al. 2000). It can be completely suppressed by RNAi with *cep-1* (Derry et al. 2001; Schumacher et al. 2001), which encodes the *C. elegans* homolog of mammalian p53. In *rad-51 dpy-13 gfp(RNAi)* we observed 16.7 ± 4.6 cell corpses, but in *rad-51 dpy-13 cep-1(RNAi)* only 1.3 ± 0.8 corpses per gonad ($n=10$ gonads each). It should be noted that the level of germ cell death in the wild-type control upon irradiation is lower than previously described (Gartner et al. 2000), which is presumably due to the *dpy-13* background, which results in slower germ cell proliferation and hence leads to a lower level of germ cell apoptosis.

Meiosis in a *rad-51* mutant

To elucidate the role of RAD-51 in meiosis, we studied chromosome behavior in the ovotestis of *rad-51(lg08701)* hermaphrodites. Cells at different meiotic stages can be

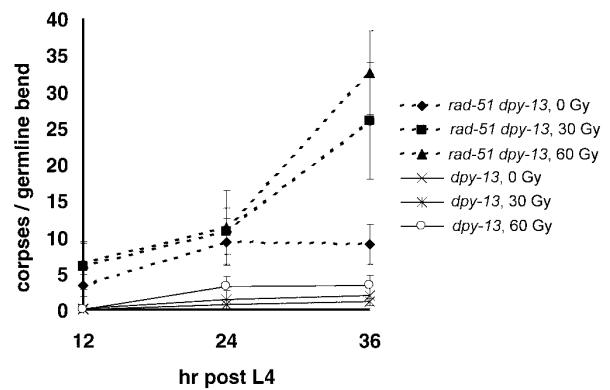


Fig. 2 Elevated germ cell apoptosis in the *rad-51(lg08701)* disruption without and after irradiation. Late stage L4 hermaphrodites were exposed to 0, 30 and 60 Gy of γ -irradiation and apoptosis was scored 12, 24 and 36 h later as described in Gartner et al. (2000). The y-axis indicates the number of dead cells (cell corpses) per gonad arm. 15–20 gonad arms were scored for each data point; error bars indicate average deviation

discriminated by their nuclear morphology after staining with the DNA-specific fluorescent dye DAPI and by their presence in easily distinguishable zones along the tubular gonad. To assess meiotic chromosome pairing in *rad-51(lg08701)* worms, the association of homologous chromosomal loci, which were labeled by FISH, was investigated. We found single FISH signals (indicative of chromosome pairing) in transition zone and early and late pachytene cells at a frequency that is comparable to wild type (Fig. 3A, B). This result indicates that homology recognition and homologous synapsis are unaffected by the absence of functional *rad-51*. In addition, immunostaining of REC-8, a component of SCs (Pasierbek et al. 2001), revealed the wild-type appearance of this structure in the *rad-51(lg08701)* mutant (Fig. 3C). These results suggest that *C. elegans* RAD-51 is required for neither presynaptic alignment, synapsis nor for the proper morphological appearance of the SC.

In most diakinesis nuclei of *rad-51(lg08701)*, chromosomes were clumped together and appeared diffuse. This phenotype is also elicited by RNAi depletion of RAD-51 (Takanami et al. 1998; Rinaldo et al. 2002; our unpublished observations) and it was interpreted as a chromosome condensation defect. Whereas, in the wild type, six well-condensed bivalents are visible at this stage (Fig. 3D), many nuclei had six weakly condensed bivalents (Fig. 3E–G). The presence of bivalents is surprising since mutants defective in meiotic recombination are expected to form 12 univalents as chiasmata that hold homologous chromosomes together would be missing. Only a subset of diakinesis nuclei revealed more than six chromatin masses, indicating a failure of chiasma formation (Fig. 3H). In very old *rad-51(lg08701)* worms (i.e., at the time when they had run out of sperm) we often observed 12 properly condensed univalents (Fig. 3I), a phenotype that has been previously observed in *spo-11*, *mre-11* and *msh-5* recombination-defective worms (Dernburg et al. 1998; Kelly et al. 2000;

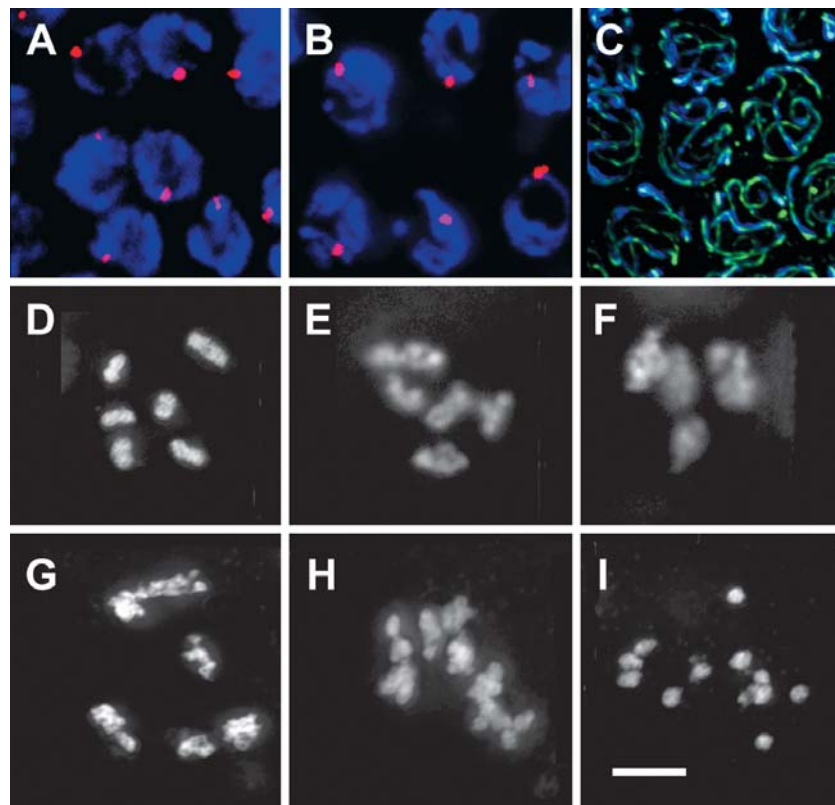


Fig. 3A–I Meiotic phenotypes of *rad-51(lg08701)*. Meiotic chromosome pairing is normal in the mutant. Fluorescence in situ hybridization (FISH) performed with a probe for a chromosomal region near the left end of chromosome *I*. Most pachytene nuclei show a single or tightly linked doublet FISH signals (*red*) both in the wild type (**A**) and the *rad-51* mutant (**B**), which indicate close alignment of homologous chromosomal regions. DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (*blue*). **C** The mutant is capable of proper synaptonemal complex (SC) assembly. Immunostaining of *rad-51(lg08701)* pachytene nuclei with the SC

component REC-8 (*green*) indicates normal SC formation at pachytene. **D–I** Chromosome morphology in wild-type and *rad-51(lg08701)* diplotene/diakinesis (DAPI staining). Whereas in the wild type (**D**) six well-condensed bivalents are formed, in most cells of the mutant corresponding to diplotene-diakinesis, one to several chromatin masses are observed, but occasionally approximately six entities, resembling bivalents, can be resolved. In a subset of nuclei at this stage, more than six structures are present. Twelve well-condensed univalents are formed in old mutant individuals (**I**). *Bar* represents 5 μ m

Chin and Villeneuve 2001). Given the variability of diakinesis phenotypes we wondered whether a small maternally provided pool of RAD-51 might persist in *rad-51(lg08701)* worms and cause variable phenotypes. We therefore performed *rad-51* RNAi in *rad-51(lg08701)* worms. Since we observed similar phenotypes to that of the deletion only (data not shown), we conclude that *rad-51(lg08701)* displays a complete loss of function phenotype with respect to diakinesis. It is possible, however, that the aforementioned second *RecA* homolog of *C. elegans* has a partially redundant function.

Immunolocalization of RAD-51 in meiotic cells of wild-type and mutant worms

To characterize further the roles of *C. elegans* RAD-51 we examined the cytological localization of RAD-51 during meiosis. Immunostaining of RAD-51 showed that it forms distinct round or slightly elongated dots within nuclei of the gonad. We only occasionally observed

RAD-51 spots in mitotic cells. No RAD-51 staining was observed in *rad-51(lg08701)* worms or when a pre-immune serum was used (data not shown). However, RAD-51 foci were readily induced in mitotic cells upon irradiation in a dose- and time-dependent manner, similar to the situation in yeast and mammalian cells (Anton Gartner and Arno Alpi, unpublished results). The number of RAD-51 spots was very heterogeneous between cells at comparable stages within one and the same gonad. In a small fraction of nuclei in the mitotic zone 1 or 2 spots occurred (data not shown). In meiotic cells RAD-51 foci appeared in nuclei of the transition zone of the gonad, which corresponds to the leptotene to zygotene stage (Fig. 4A, Table 1). We found that many of the RAD-51 spots were bar-shaped (Fig. 4A, and insert). This observation is in accordance with the bar-shaped or double Rad51p foci in maize (Franklin et al. 1999). They were interpreted as Rad51p nucleofilaments bridging the distance between two aligned homologs in the course of strand invasion or another recombination intermediate. In nuclei immediately adjacent to the transition zone (which

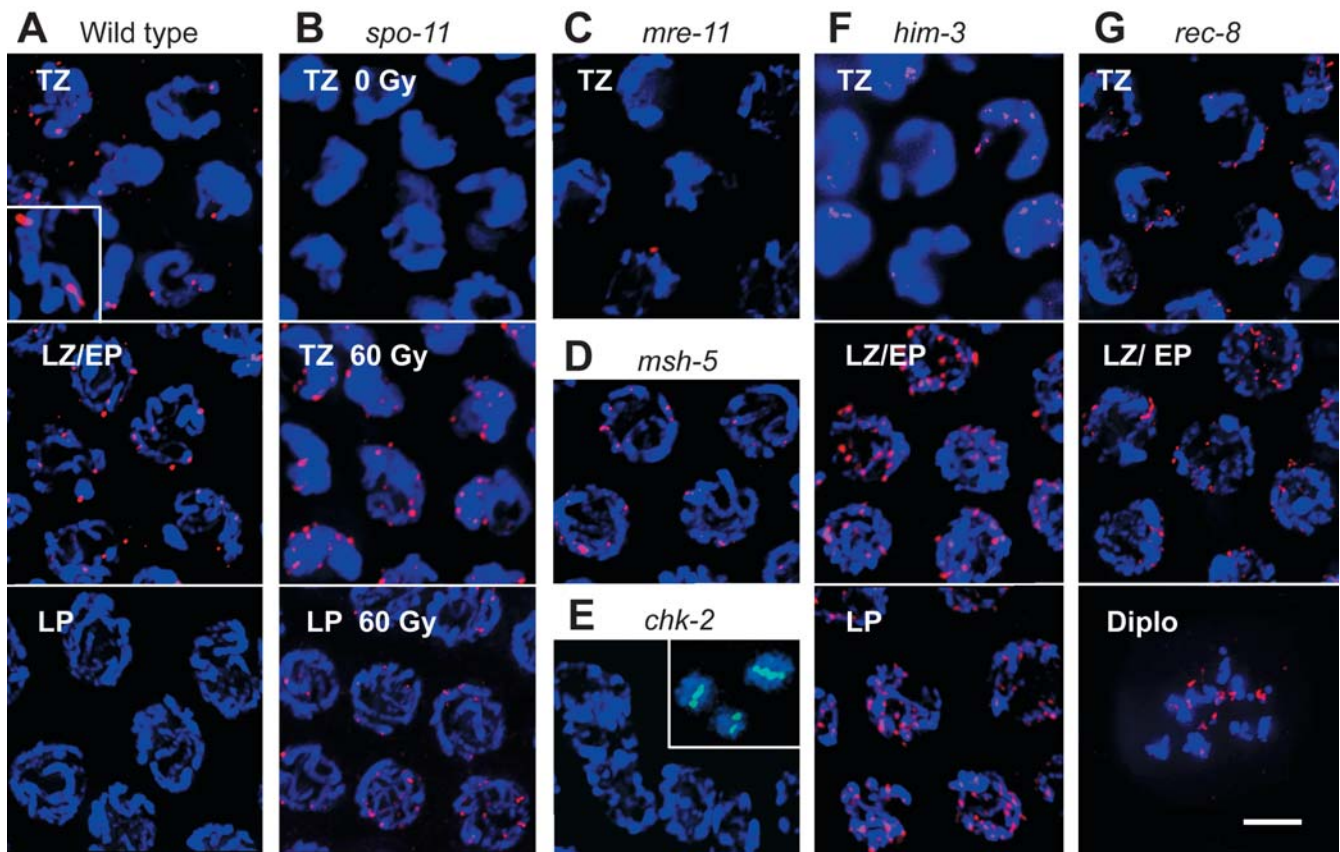


Fig. 4A–G Localization of RAD-51 in meiotic nuclei. **A** Immunostaining highlights distinct chromatin-associated spots (red) in wild-type transition zone (TZ) and late zygotene/early pachytene nuclei (LZ/EP). They are absent from late pachytene nuclei (LP). Many of the RAD-51 spots are bar-shaped (insert). **B** Rad-51 staining of transition zone nuclei in unirradiated (0 Gy) and transition zone and late pachytene nuclei in irradiated (60 Gy) *spo-11* worms. **C** RAD-51 staining of *mre-11* transition zone nuclei. **D**

RAD-51 staining in *msh-5* late pachytene nuclei. **E** Rad-51 foci do not form in *chk-2* worms. Insert At a later stage well condensed univalents are present whose axes contain REC-8 (green). **F** Examples of RAD-51 foci in *him-3(RNAi)* worms in transition zone, late zygotene/early pachytene and late pachytene nuclei. **G** RAD-51 foci in *rec-8(RNAi)* worms occur from transition zone to diplotene (Diplo). For quantitative data see Table 1. Bar represents 5 μ m

Table 1 Quantification of RAD-51 foci in several genetic backgrounds and meiotic prophase stages of *Caenorhabditis elegans*. Average numbers \pm SD of RAD-51 foci per nucleus are listed, and the minimum–maximum number per nucleus and the percentage of nuclei showing at least one RAD-51 focus are given in parentheses. The percentages of nuclei in which RAD-51 foci occurred in

distinct clusters in the translocation strains (for examples see Fig. 5B) are given in square brackets. Late zygotene/early pachytene nuclei were classified by their position in three to four rows of cells adjacent to the transition zone. Classification of transition and pachytene zone was according to MacQueen and Villeneuve (2001)

| | Transition zone (i.e., leptotene-zygotene) | Late zygotene/early pachytene zone | Mid/late pachytene zone |
|--|---|---|---|
| Wild type | 0.9 \pm 1.0, n =56 (0–6, 48%) | 5.2 \pm 1.7, n =44 (1–11, 100%) | <0.1, n =38 (0–1, 10%) |
| <i>rad-51(lg08701)</i> | 0, n =40 | 0, n =44 | 0, n =41 |
| <i>spo-11(ok79)</i> | <0.1, n =67 (0–1, 3%) | <0.1, n =36 (0–1, 3%) | 0, n =42 |
| <i>spo-11(ok79)-</i> Irradiated 60 Gy | 13.9 \pm 3.4, n =41 (6–25, 100%) | 21.2 \pm 3.8, n =57 (12–29, 100%) | 11.3 \pm 3.1, n =38 (3–17, 100%) |
| <i>chk-2(me64)</i> | <0.1, n =35 (0–1, 3%) | <0.1, n =60 (0–1, 3%) | 0, n =39 |
| <i>mre-11(ok179)</i> | <0.1, n =35 (0–1, 3%) | <0.1, n =50 (0–1, 3%) | 0, n =50 |
| <i>msh-5(me23)</i> | 0.5 \pm 0.6, n =48 (1–13, 58%) | 4.0 \pm 1.8, n =40 (1–10, 100%) | 3.2 \pm 1.4, n =59 (1–7, 91%) |
| <i>him-3(RNAi)</i> | 3.6 \pm 4.5, n =38 (0–20, 53%) | 18.7 \pm 4.9, n =41 (9–35, 100%) | 17.2 \pm 3.1, n =40 (10–33, 100%) |
| <i>rec-8(RNAi)</i> | 2.6 \pm 3.0, n =52 (0–15, 58%) | 13.9 \pm 2.8, n =41 (9–21, 100%) | 13.0 \pm 2.8, n =40 (8–21, 100%) |
| <i>rec-8(cosuppression)</i> | 1.6 \pm 1.5, n =50 (0–6, 66%) | 12.7 \pm 3.4, n =41 (5–18, 100%) | 24.5 \pm 5.7, n =40 (11–35, 100%) |
| <i>eT1(II;V)</i> | 1.6 \pm 1.6, n =58 (0–10, 59%) | 8.0 \pm 2.7, n =43 (3–16, 100%) [62%] | 8.2 \pm 3.3, n =44 (0–20, 93%) [75%] |
| <i>hT1(I;V)</i> | 1.2 \pm 1.2, n =47 (0–6, 63%) | 8.5 \pm 3.1, n =42 (2–18, 100%) [65%] | 10.1 \pm 4.0, n =56 (0–20, 96%) [74%] |

correspond to late zygotene/early pachytene) up to nine RAD-51 spots were present, whereas by late pachytene they were gone (Fig. 4A).

It has been demonstrated that *C. elegans spo-11* is required to generate meiotic DSBs (Dernburg et al. 1998). To determine whether *spo-11*-dependent breaks are the only targets of RAD-51 in *C. elegans* we analyzed the pattern of RAD-51 foci in *spo-11* worms. We found that there were virtually no RAD-51 foci in any meiotic stage (Table 1), indicating that RAD51 associates with DSBs only. Irradiation bypasses the recombination defect of *spo-11* worms, leading to the establishment of bivalents due to the formation of functional chiasmata (Dernburg et al. 1998). As expected, this bypass of *spo-11* function likely depends on *rad-51* as irradiated *spo-11* worms displayed RAD-51 foci (Fig. 4B).

Likewise, we scored RAD-51 in an *mre-11(ok179)* mutant. While synapsis is normal in this mutant, it shows a meiotic recombination defect. It was proposed that, similar to the situation in yeast, *C. elegans* MRE-11 may have a dual role in DSB generation and processing (Chin and Villeneuve 2001). In accordance with the putative function of *mre-11* in the generation of meiotic DSBs or in the generation of ssDNA via the resection of DSBs, we only observed background levels of RAD-51 foci in meiotic nuclei of the mutant (Table 1, Fig. 4C).

We next studied RAD-51 foci in the *msh-5(me23)* mutant, which has a normal SC but which is defective in chiasma formation (Kelly et al. 2000). *msh-5* encodes the *C. elegans* MutS homolog and genetic evidence indicates that it might act after the initiation of meiotic recombination (Kelly et al. 2000). We found wild-type levels of RAD-51 foci in transition zone nuclei and in early pachytene nuclei of *msh-5(me23)*. However, these foci persisted longer as compared with wild-type cells and could also be found in late pachytene cells (Fig. 4D). This result suggests a defect in the processing of recombination intermediates that occurs after RAD-51 focus formation and that leads to the accumulation of RAD-51 foci in pachytene. Foci completely disappeared in diplotene, indicating that DSBs eventually become repaired or that RAD-51 dissociates from breaks.

To investigate the link between DSBs, presynaptic alignment, SC formation and RAD-51 focus formation, we performed RAD-51 immunostaining in several meiotic pairing mutants. First, we checked whether RAD-51 is present on chromatin in a *chk-2* mutant. In this mutant presynaptic alignment and SC formation is compromised and transition zone nuclei appear abnormal whereas other aspects of meiosis are normal and 12 well-condensed univalents are formed (MacQueen and Villeneuve 2001). These univalents possess axes that contain the meiosis-specific cohesin REC-8 (Fig. 4E, insert). It is not known whether meiotic recombination is initiated in the absence of presynaptic alignment in *chk-2* (MacQueen and Villeneuve 2001). No RAD-51 foci were found in this mutant (Table 1, Fig. 4E), suggesting that DSBs either do not occur or are not processed to a recombination intermediate that is a target of RAD-51.

We next studied the effect of HIM-3 depletion by RNAi on RAD-51 focus formation. HIM-3 localizes to chromosomal cores, and in *him-3* hypomorphs both meiotic recombination and synapsis are severely reduced (Zetka et al. 1999). Analysis by FISH confirmed that, upon dsRNA injection (which does not necessarily lead to the complete depletion of HIM-3), synapsis was abolished (data not shown). In *him-3(RNAi)* worms the incidence of RAD-51 foci was dramatically increased and a large number of RAD-51 foci persisted even in pachytene cells (Table 1, Fig. 4F), suggesting that DSBs are formed abundantly on chromosomes that are not synapsed. Interestingly, we did not observe an elevated level of programmed cell death in *him3(RNAi)* worms. There was an average of 0.88 to 1.2 corpses per gonad in four independent *him-3* RNAi depleted worm lines, which is similar to the amount of dead cells in the wild type (Gartner et al. 2000). This result indicates that in *C. elegans* programmed pachytene cell death is not triggered by synaptic failure or by the large excess of DSBs in *him3* worms. Since RAD-51 spots disappeared in diplotene, we assume that DSBs are eventually repaired even in the absence of synapsis or that RAD-51 protomers finally dissociate from the sites of breaks.

To corroborate further the finding that synapsis is required to remove RAD-51 spots, we analyzed the cytological behavior of RAD-51 foci in *rec-8(RNAi)* and *rec-8(cosuppression)* worms. *rec-8* encodes a meiotic cohesin that is localized at chromosomal axes and between sister chromatids. Depletion of REC-8 by RNAi leads to a defect in SC formation, whereas presynaptic alignment is maintained. Furthermore, it was suggested that meiotic DSBs remain unrepaired in *rec8* as chromosomal fragments can be detected at diplotene (Pasierbek et al. 2001). To see whether RAD-51 foci persist at unrepaired DSBs, we investigated the number of RAD-51 foci in *rec-8(RNAi)* and *rec-8(cosuppression)* worms. The number of RAD-51 spots in the transition zone was increased and a dramatically increased number of spots persisted into pachytene (Table 1). RAD-51 foci remained associated with chromatin even up to the late diplotene stage (Fig. 4G). Only at diakinesis did the RAD-51 foci disappear, which suggests that DSBs are repaired by a mechanism that does not require the sister or the homolog as template or that they remain unrepaired but RAD-51 dissociates from these sites after some time.

To exclude the possibility that RAD-51 foci persist in the absence of HIM-3 and REC-8 owing to compromised axial element structure rather than owing to genuine defects in meiotic synapsis, we abolished synapsis by inactivating *syp-1*, which encodes a structural element of the transverse filaments of the SC (MacQueen et al. 2002). Consistent with the notion that SC formation is required for the removal of RAD-51 foci, we found that numerous RAD-51 foci persist in the nuclei of the late pachytene zone of *syp-1(RNAi)* animals (data not shown). The *him-3*, *rec-8* and *syp-1* deficiency situations demonstrate that in the absence of synapsis RAD-51 remains associated with chromatin.

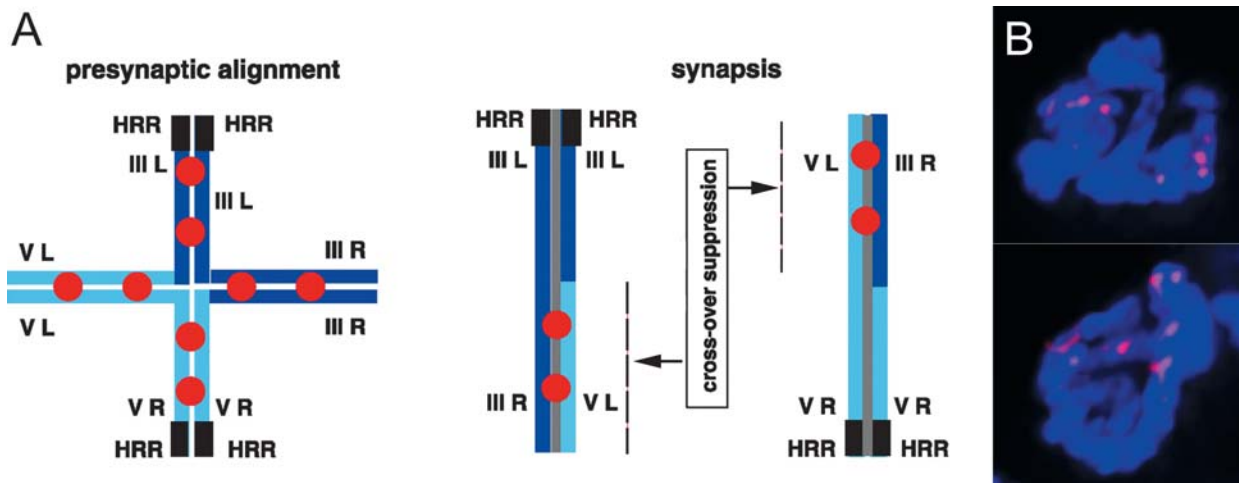


Fig. 5A, B RAD-51 foci persist in heterozygous translocation strains. **A** Model of chromosome pairing during presynaptic alignment and synapsis in the *eT1* translocation. During presynaptic alignment homologous DNAs associate irrespective of whether they are on homologous chromosomes or not. Synapsis starts from

homologous recognition regions (*HRR*) and extends into non-homologous regions that are crossover suppressed. RAD-51 foci are indicated as *red dots*. **B** Two examples of late pachytene cells in worms bearing heterozygous *eT1* translocations

To test further whether SC formation is sufficient to remove RAD-51, we employed the heterozygous translocation strain *eT1 (III;V)*. In this strain, the right arm of chromosome *III* and the left arm of chromosome *V* are reciprocally exchanged (Edgley et al. 1995). Studies employing FISH probes indicated that presynaptic alignment occurs between homologous sequences even if those sequences are on separate chromosomes as is the case in the *eT1 (III;V)* translocation (Loidl et al. 2003; for illustration see Fig. 5A). Subsequently SC formation is initiated from the non-translocated part of homologous chromosomes and non-homologous SCs are possibly formed at the translocated areas of the affected chromosomes (Loidl et al. 2003; A. Dernburg and A. Villeneuve, personal communication). Consequently, two unsynapsed or non-homologous SC tracts are present in pachytene nuclei within the reciprocally translocated areas of chromosomes *III* and *V*. Immunostaining with RAD-51 revealed the presence of numerous RAD-51 foci (Table 1), which tended to be arranged in a string-like or clustered arrangement in two distinct areas of the nucleus (Fig. 5B). These results suggest that RAD-51 foci persist in unsynapsed or in non-homologously synapsed regions of the genome. The results obtained with the analysis of *eT1 (III;V)* were also confirmed by the analysis of another reciprocal translocation, *hT1(I;V)* (Table 1).

Discussion

RAD-51 protein accumulates in spots in nuclei undergoing DSB repair

We found that ~0–11 distinct RAD-51 foci occur in transition zone nuclei (corresponding to leptotene/zygotene) and in early pachytene nuclei of wild-type worms.

By late pachytene, all RAD-51 foci were gone (Table 1). The presence of RAD-51 in early meiotic prophase nuclei is in agreement with the established role of the protein in the processing of meiotic DSBs (Paques and Haber 1999) that occurs around the zygotene stage in yeast (Allers and Lichten 2001; Hunter and Kleckner 2001). No RAD-51 spots were present in DSB-deficient *spo-11* mutant worms, confirming evidence from other organisms that RAD-51 protein is associated with meiotic DSBs only (for review see Masson and West 2001). Similarly, we did not find RAD-51 spots in *mre-11* mutants, confirming that *C. elegans* MRE-11 is required to produce or to process meiotic DSBs. The timing of RAD-51 focus formation resembles the situation in budding yeast where Rad51 dissociates from chromosome axes during incipient SC formation (Bishop 1994). Similarly, immunolabeling of RAD-51 in a variety of organisms has in general revealed similar dynamics: appearance in leptotene or zygotene, maximal abundance in zygotene, and reduction in pachytene (Ashley et al. 1995; Terasawa et al. 1995; Plug et al. 1996; Barlow et al. 1997a; Moens et al. 1997; Franklin et al. 1999; Tarsounas et al. 1999).

RAD-51 foci are remarkably rare in *C. elegans* wild-type meiosis

In budding yeast, mouse, lily and maize, several tens to hundreds of RAD-51 foci were counted in leptotene to early pachytene nuclei where they decorate the axes of unsynapsed chromosomes (Bishop 1994; Ashley et al. 1995; Terasawa et al. 1995; Franklin et al. 1999; Tarsounas et al. 1999). In lily and mouse it was shown that RAD-51 foci colocalize with early nodules (Anderson et al. 1997; Tarsounas et al. 1999) that mark the sites of first convergence of homologs undergoing presynaptic

alignment (Albini and Jones 1987; Stack et al. 1993). In *Saccharomyces cerevisiae* it has been proposed that the axial elements of homologs converge and synapsis initiates at the sites of interchromosomal recombination (Chua and Roeder 1998; Agarwal and Roeder 2000). The observed number of these sites (~57 as scored by the localization of Zip3p; Agarwal and Roeder 2000) corresponds well with the maximum number (64) of RAD-51/Dmc1p foci (Bishop 1994) although both are less frequent than the estimated mean number of 75–90 crossovers per nucleus (Jacobson et al. 1975; Agarwal and Roeder 2000), not to speak of recombination events if non-crossover outcomes are included. However, this difference could be explained by the tendency of RAD-51 foci to fuse and/or by the asynchronous occurrence of RAD-51 foci.

Compared with the organisms mentioned, the number of meiotic RAD-51 foci per nucleus (0–11) is remarkably low in *C. elegans*, and on average there may be less than one focus per chromosome that can be observed at any given time. Asynchronous formation or conglomeration of DSBs/RAD-51 foci may only partially explain their sparseness. It is tempting to speculate that in *C. elegans*, since there is no requirement for DSBs to promote SC formation, only relatively few DSBs occur in addition to those that are resolved as crossovers and result in the six chiasmata per cell. On the other hand, we observed up to 35 RAD-51 spots in synapsis-defective mutants and up to 20 RAD-51 spots in translocation strains where approximately 20% of the genome does not form homologous SCs. This increased number of RAD-51 foci might be due to the accumulation of RAD-51 foci in the continued absence of synapsis (see below).

RAD-51 foci are removed from homologously synapsed chromosomes

RAD-51 foci accumulate in pachytene cells that are incapable of homologous synapsis due to the depletion of *him-3*, *rec-8* and *syp-1* (Zetka et al. 1999; Pasierbek et al. 2001; MacQueen et al. 2002). In addition, it has been shown for *him-3* and *syp-1* hypomorphs that they are severely defective in meiotic recombination (Zetka et al. 1999; MacQueen et al. 2002). These results are in accord with previous experiments from budding yeast where RAD-51 foci persist in mutants devoid of an SC (Bishop 1994). Our data also demonstrate that meiotic DSBs originate independently of SC formation and they are compatible with the evidence derived from other organisms that in the wild type DSB formation and processing by a RAD-51-dependent pathway precede synapsis (Bishop 1994). In addition, the appearance of RAD-51 foci in leptotene/zygotene nuclei in wild-type *C. elegans* confirms that DSB formation occurs prior to chromosome synapsis, although it is not a precondition for the latter in this organism (Dernburg et al. 1998).

RAD-51 spots, mostly arranged in clusters, also persisted into pachytene in the two heterozygous translocation strains investigated. Crossing over does not occur

in the translocated chromosome regions, for which reason they are widely used as genetic balancers (Edgley et al. 1995). We suspect that RAD-51 spots remain attached to the affected arms because they do not synapse homologously. There is evidence from electron microscopy that synapsis, which is initiated in the homologous (homology recognition region-bearing) portions of translocation chromosomes, continues into the adjacent non-homologous regions, since Goldstein (1986) reported the presence of six linear, unforked SCs in a heterozygous translocation strain. This raises the possibility that some of the regions where RAD-51 foci persist are non-homologously synapsed, which would mean that synapsis needs to be homologous for RAD-51 foci to disappear. Thus, our experiments suggest that within the framework of homologous SCs, DSBs are subject to recombinational repair and are no longer a substrate of RAD-51.

While RAD-51 foci form in mutants that do not initiate synapsis, we did not observe RAD-51 foci in the *chk-2* mutant where initial homology recognition (as indicated by presynaptic alignment) fails (MacQueen and Villeneuve 2001). Thus, unlike in budding yeast where the initiation of recombination is independent of the interaction of homologs (de Massy et al. 1994; Gilbertson and Stahl 1994), successful homology recognition might be a precondition for DSB formation or RAD-51 recruitment to DSBs in *C. elegans*.

Double-strand breaks do not necessarily trigger apoptosis

Our *rad-51* deletion mutant displayed an elevated level of germ cell death, which triggers the DNA damage-dependent, p53-suppressible apoptotic pathway, presumably as a consequence of unrepaired meiotic DSBs. Thus, similar to mammalian systems, meiotic failure can trigger a DNA damage checkpoint pathway that requires p53 for the activation of programmed cell death (Barlow et al. 1997b). On the other hand, in *him-3(RNAi)* and *rec-8(RNAi)* synapsis-defective worms and in worms bearing translocation chromosomes, where RAD-51 foci accumulate, programmed cell death is not elicited. These results imply that the *C. elegans* pachytene checkpoint does not respond to all DSBs. Furthermore, our results suggest that the pachytene checkpoint does not sense the failure to synapse homologous chromosomes properly.

Depletion of RAD-51 causes diffuse chromatin and univalent formation in diakinesis

Analysis of diakinesis nuclei of *rad-51* mutants revealed a variable phenotype mostly comprising diffuse masses of chromatin that were difficult to tell apart. This phenotype was similarly observed by the depletion of *rad-51* by RNAi (Takanami et al. 1998; Rinaldo et al. 2002). Quite often six diffuse masses of chromatin could be discerned, indicating the formation of bivalents. This result is remarkable, because the *rad-51(lg08701)* allele has to

be considered a null mutation and the phenotype of *rad-51(lg08701)* could not be enhanced by *rad-51(RNAi)*. After the disappearance of the SC at the end of pachytene, homologous contacts are believed to rely on the exchange of DNA strands at incipient chiasmata. The occurrence of bivalent-like structures in *C. elegans* suggests that, whatever kind of DNA interactions are taking place in the absence of RAD-51, they are sufficiently robust to maintain the physical association between the majority of homologs. Although the diffuse appearance of diakinesis chromatin has been interpreted as a condensation defect, it possibly could also arise due to massive DNA fragmentation that occurs due to the generation of many meiotic DSBs that cannot be repaired in *rad-51* mutants. This notion is supported by the recent finding of Rinaldo et al. (2002), according to which the diffuse appearance of pachytene chromatin is suppressed in *rad51(RNAi) spo-11* and *rad51(RNAi) mre-11* strains, which contain nicely condensed univalents, presumably because they fail to generate meiotic DSBs. Furthermore, irradiated diakinesis nuclei of *mre-11* worms look similar to *rad-51*, likely because breaks that are generated upon irradiation cannot be repaired (Chin and Villeneuve 2001). If one assumes that many *spo-11*-dependent DSBs per chromosome are generated during meiosis, one can also explain the 100% lethality we observed in the progeny of *rad-51* worms. In this case, embryonic lethality would not only be caused by aneuploidy due to random meiotic chromosome segregation (that would by chance result in rare surviving embryos with a correct chromosome set), but also by abundant chromosome breaks that are generated by SPO-11 but cannot be repaired properly in the absence of RAD-51. Since we occasionally observed 12 nicely formed univalents in very old *rad-51* worms, we speculate that in old worms where oocytes are not fertilized anyway, the incidence of SPO-11-generated DSBs is reduced.

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