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An essential role of DmRad51/SpnA in DNA repair and meiotic checkpoint control

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Rad51 is a conserved protein essential for recombinational repair of double-stranded DNA breaks (DSBs) in somatic cells and during meiosis in germ cells. Yeast Rad51 mutants are viable but show meiosis defects. In the mouse, RAD51 deletions cause early embryonic death, suggesting that in higher eukaryotes Rad51 is required for viability. Here we report the identification of SpnA as the *Drosophila* Rad51 gene, whose sequence among the five known *Drosophila* Rad51-like genes is most closely related to the Rad51 homologs of human and yeast. *DmRad51/spnA* null mutants are viable but oogenesis is disrupted by the activation of a meiotic recombination checkpoint. We show that the meiotic phenotypes result from an inability to effectively repair DSBs. Our study further demonstrates that in *Drosophila* the Rad51-dependent homologous recombination pathway is not essential for DNA repair in the soma, unless exposed to DNA damaging agents. We therefore propose that under normal conditions a second, Rad51-independent, repair pathway prevents the lethal effects of DNA damage.

Keywords: *Drosophila*/DSB repair/meiosis/Rad51/spindle-A

Introduction

Chromosomal integrity is essential for proper embryonic and postembryonic development, prolonged survival and successful reproduction. Highly conserved repair mechanisms exist in all organisms, from bacteria to mammals, to recognize and repair DNA damage (Sancar, 1996; Wood, 1996; Sekelsky *et al*., 2000; Wood *et al*., 2001). The repair of double-stranded DNA breaks (DSBs) is a necessary mechanism for recombining parental genomes during meiosis and is used as a defense mechanism after DNA damage caused by irradiation or chemical agents. The presence of DNA damage activates a cell cycle checkpoint (Melo and Toczyski, 2002). This allows time for the cell to correct the damage so as not to propagate the defect or affect normal cellular functions. In *Saccharomyces cerevisiae* mutations in the same genes show increased sensitivity to ionizing radiation and meiotic phenotypes, such as chromosome non-disjunction and/or rearrangements (Symington, 2002). This suggested a functional relationship between the mechanisms of mitotic DNA repair and meiotic recombination.

Genetic studies in *S.cerevisiae* led to the discovery of the Rad52 epistasis group of DSB repair genes (Symington, 2002). A core protein in this pathway is Rad51, which is related to the bacterial RecA protein. Rad51 has DNA-dependent ATPase activity and catalyzes strand exchange between homologous DNA molecules. Rad51 and Rad51-related proteins are found from yeast to humans (Shinohara and Ogawa, 1999; Thacker, 1999). In yeast, the Rad51 null mutant is viable but shows sporulation defects (Shinohara *et al*., 1992). The mouse Rad51 knockout is embryonic lethal (Lim and Hasty, 1996; Tsuzuki *et al*., 1996), thus the role of Rad51 in mouse meiosis could not be studied. In both mouse and yeast, a meiosis-specific Rad51-related gene, Dmc1, has been identified and shown to be required for chromosome synapsis and strand exchange during prophase of meiosis I (Bishop *et al*., 1992; Pittman *et al*., 1998).

In contrast to yeast, *Drosophila* members of the Rad52 epistasis group were not identified on the basis of meiosis defects or mutagen sensitivity. Rather, mutations in the *Drosophila* RAD51-related gene, *spindle-B* (*spnB*), and the Rad54 homolog, *okra*, were discovered as maternal-effect mutants with altered patterning of the eggshell, the so-called *spindle* phenotype (Morris and Lehmann, 1999). It was shown that this phenotype, observed in *spnB* and *okra* mutants, was due to reduction in the levels of the morphogen Gurken, a TGFα-like protein that controls both dorso–ventral patterning of the egg and antero–posterior polarity of the embryo (Ghabrial *et al*., 1998). Schüpbach and colleagues suggested that the activation of a meiotic checkpoint, which resulted in defective Gurken translation, was the result of a failure to repair DNA breaks in mutants for *okra*, *spnB* and *spindle-D* (*spnD*), another Rad51-related protein (Ghabrial and Schüpbach, 1999; Abdu *et al*., 2003). Accordingly, the *spindle* phenotype was suppressed by mutants for the Spo11 homolog, *mei-W68*, which are defective in double-stranded break formation and thus are unable to activate the checkpoint (Ghabrial and Schüpbach, 1999). *Spn* mutants were also suppressed in combination with mutants of known transducers of cell cycle checkpoints, such as *Drosophila mei-41*, an ATR/ATM phosphatidylinositol 3-kinase-like protein, and the *Drosophila* homolog of Chk2 kinase, *chk2/mnk/loki* (Ghabrial and Schüpbach, 1999; Abdu *et al*., 2002). A target for the meiotic checkpoint in *Drosophila* is the ATP-dependent helicase Vasa (Styhler *et al*., 1998; Tomancak *et al*., 1998), which is phosphorylated upon checkpoint activation and may regulate Gurken translation (Ghabrial and Schüpbach, 1999). Sequence analysis

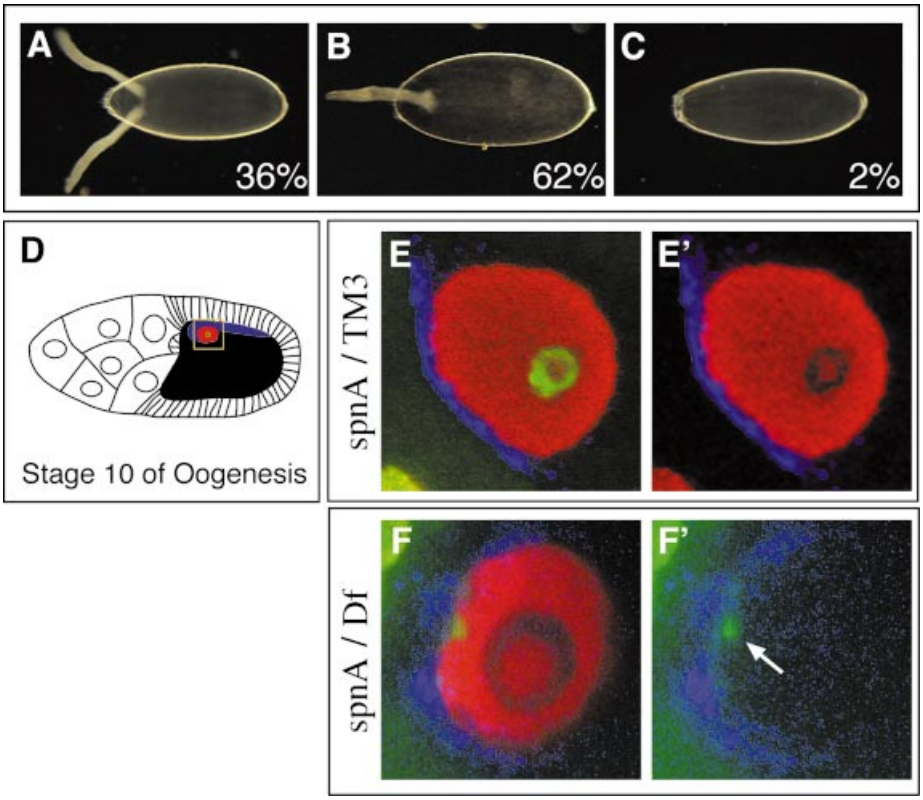


Fig. 1. *spnA* mutant phenotypes. (A–C) Eggshell phenotype. (**A**) Wild-type with two anteriorly located dorsal appendages. (**B**) Mild ventralization with a single fused-dorsal appendage. (**C**) Completely ventralized. Percentages represent the phenotypic proportions observed per total eggs laid (n = 374) from $spnA^{155–52}$ germline clones. (D–F′) Gurken expression and karyosome phenotype. (**D**) Diagram of a wild-type Stage 10 egg chamber. The oocyte nucleus is indicated in red, Gurken protein in blue, karyosome in green. (**E** and **E′**) A confocal section of a germinal vesicle from a $spnA^{093A}$ heterozygote Stage 10 egg chamber. Staining for the centrosomal protein, CP190 (red; Whitfield *et al*., 1995), shows the nucleoplasm. (**F** and **F′**) Germinal vesicle from a $spnA^{093A}$/*Df(3R)X3F* Stage 10 egg chamber. (E′) is identical to (E) minus the green channel. (F′) is identical to (F) minus the red channel. The yellow box in (D) represents the area captured in (E) and (F). [(A–C) dark-field photographs using 20× objective, anterior to the left; (E and F) confocal images using a 40× objective and 4× zoom, dorsal view with anterior to the left].

indicates that there are at least five *Drosophila* genes that show significant homology to yeast and human Rad51 (this report). It remained unclear whether these genes have distinct functions in DSB repair and whether the activation of the meiotic checkpoint was a consequence of the failure to repair DSBs. Furthermore, while mutations in *spnB* showed meiotic defects, they did not affect DNA repair in somatic cells (Ghabrial *et al*., 1998), raising the possibility that in *Drosophila* distinct sets of Rad51-like genes may control DSB repair either in the germline or in the soma.

Here we report the identification of *spnA* as the *Drosophila* Rad51 gene, whose sequence among the five known *Drosophila* Rad51-like genes is most closely related to the Rad51 homologs of human and yeast. We show that *spnA* mutants exhibit the *spindle* eggshell phenotype. In *spnA* oocytes synapse of homologous chromosomes is correctly initiated during meiosis but its resolution is delayed and unrepaired double-stranded breaks persist longer than in wild type causing the activation of a meiotic recombination checkpoint. *spnA* null mutants are viable but show sensitivity to irradiation, suggesting that SpnA acts in the soma but that other repair mechanisms compensate in the absence of SpnA. Analysis of the expression pattern of the five known *Drosophila* Rad51 homologs together with the analysis of the mutant phenotype of three of these genes suggest that the *Drosophila* Rad51 genes act in concert during oogenesis and that only a subset of them are used for repair in the soma.

Results

spindle-A encodes a Drosophila Rad51 ortholog

We identified 22 new alleles of *spindle-A* by mutagenesis screening (see Supplementary data available at *The EMBO Journal* Online). *spnA* was originally identified as a maternal-effect lethal mutation affecting egg patterning (Tearle and Nusslein-Volhard, 1987). All new *spnA* alleles are viable *in trans* to the original *spnA* alleles ($spnA^{003}$, $spnA^{050}$ and $spnA^{057}$) and *in trans* to a deficiency for the region (see below), suggesting that they are not affecting a function essential for viability. Like the original *spnA* alleles, the new alleles show ~100% maternal-effect embryonic lethality. All mutant lines produce a spectrum of eggshell ventralization phenotypes similar to those described for known mutations affecting the EGFR signaling pathway, ranging from fused dorsal appendages (Figure 1B) to complete ventralization (Figure 1C; Gonzalez-Reyes *et al*., 1997). For most *spindle*-class mutants, the eggshell phenotype has been attributed to a defect in RNA and protein localization or protein synthesis of the EGFR ligand Gurken (Grk) (Gonzalez-Reyes *et al*.,

1997). In *spnA* mutants, the level and distribution of Grk protein is disrupted (Figure 1F and F′; Gonzalez-Reyes *et al*., 1997). In one example, instead of the normal crescent of Grk protein along the dorsal-anterior side of the wild-type oocyte nucleus facing the somatic follicle cells (Figure 1E and E′), Grk protein distribution is less coherent and only found in a few spots along the mutant oocyte nucleus. Another common feature shared among the *spindle*-class mutants is a disruption in oocyte nuclear morphology (Gonzalez-Reyes *et al*., 1997; Ghabrial *et al*., 1998). Mature wild-type oocytes contain highly compact chromatin called the karyosome (Figure 1E and E′). In *spnA* mutant oocytes, as well as in other *spindle*-class mutants, the DNA is less organized and diffuse. In contrast to the wild type, where DNA is found as a condensed sphere in the center of the nucleus, DNA in *spnA* mutant oocytes clusters along the periphery of the nucleus adjacent to the nuclear membrane (Figure 1F and F′).

We mapped *spnA* to the cytological region 99D01–99E01 (Supplementary figure 2). The Berkley Drosophila Genome Project predicted a Rad51-like gene (CG7948) to reside within this region of the genome. CG7948 shows strong sequence similarity to the yeast and mammalian Rad51 gene (see below). Since two other *spindle*-class genes, *spnB* and *okra*, encode members of the *Drosophila* Rad51 family and Rad54, respectively (Ghabrial *et al*., 1998), CG7948 was a likely candidate for *spnA*. Sequence analysis of *spnA* alleles revealed unique missense mutations within CG7948 (Table I). Thus, *spindle-A* encodes a *Drosophila* Rad51-like gene.

Table I. DmRAD51 allele characterization and comparison

SpnA alleles	*Drosophila* Rad51	*Saccharomyces cerevisiae* Rad51[a]	*Escherichia coli* RecA[a]
091B	L38F	L99	
050A	L57Q	L118	
093A	Q70Stop	K131	
148-15	G112D	G173	G54
104-7	E115V	E176	P57
050	S118F	S179	R60
087A	E125L	E186	P67
009A			
032B	R127H	R188	S69
119A	A138T	A199	I80
003	G149D	G210	
084A	G150S	G211	R85
068A	E151L	E212	E86
001A	R167C	R228	Y103 K106
095A	R190C	R251	G122
057	A205V	A266	A133
010A	V218M	V279	V143
061A	D219N	D280	D144
155-52	L235P	L296	I159
009D	L249F	L310	A174
091C	A253V	A314	A179
128-53	V260M	V321	L189
048B	Q265Stop	Q326	Q194
055A	G284N	G346	G211
116C	R295W	R357	R222

Tubingen screen (original) alleles are in bold italic; 3R maternal-effect screen alleles are in italic; DmRad51 allele screen alleles are in roman.
[a]Amino acid comparison based on alignment with ScRad51 and RecA (McKee *et al*., 1996).

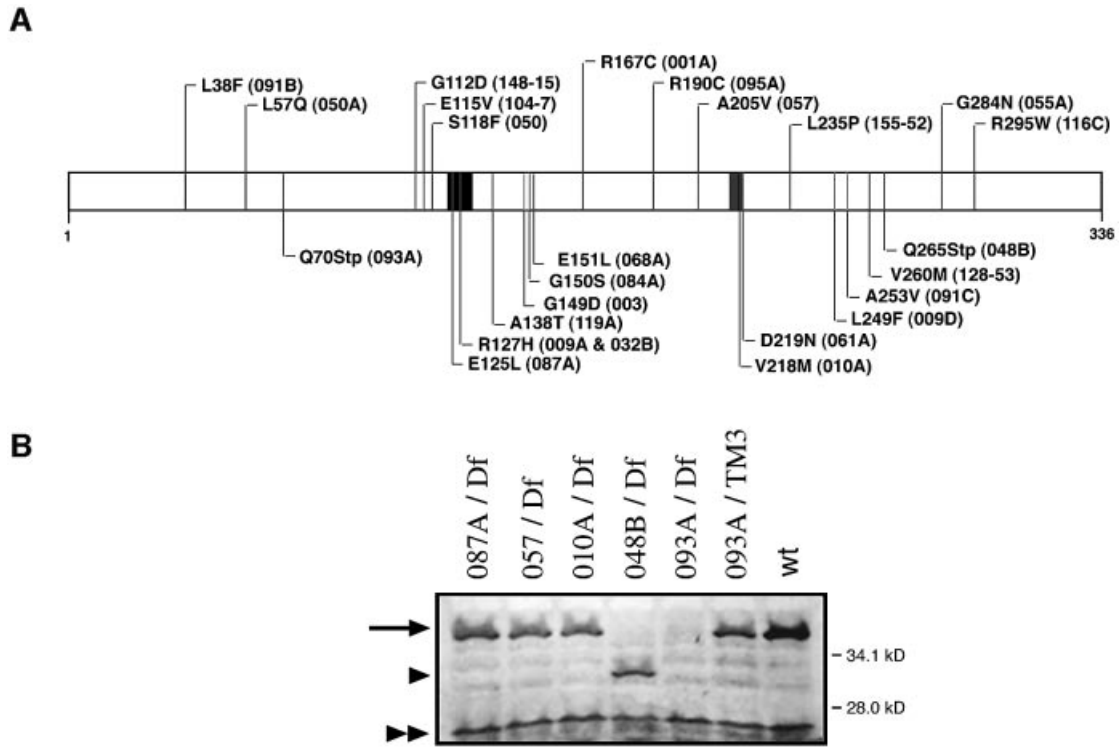


Fig. 2. Molecular characterization of the *spnA* alleles and DmRad51 protein expression analysis of mutant ovary extracts. (**A**) Map of the DmRad51 mutations. Amino acid substitution and allele name are given (in parentheses). (**B**) Western blots of extracts prepared from wild-type and hemizygote mutant ovaries. Walker-A and -B box motifs are indicated by black rectangles. Both the wild-type (wt; Oregon R) and heterozygote line, $spnA^{093A}$/*TM3*, show expression of a 36.6 kDa protein (arrow). Band shift (arrowhead) in $spnA^{048B}$/*Df(3R)X3F* ovary extracts confirms the specificity of the antibody. The lower running band (double arrowhead) is a non-specific cross-reactive protein.

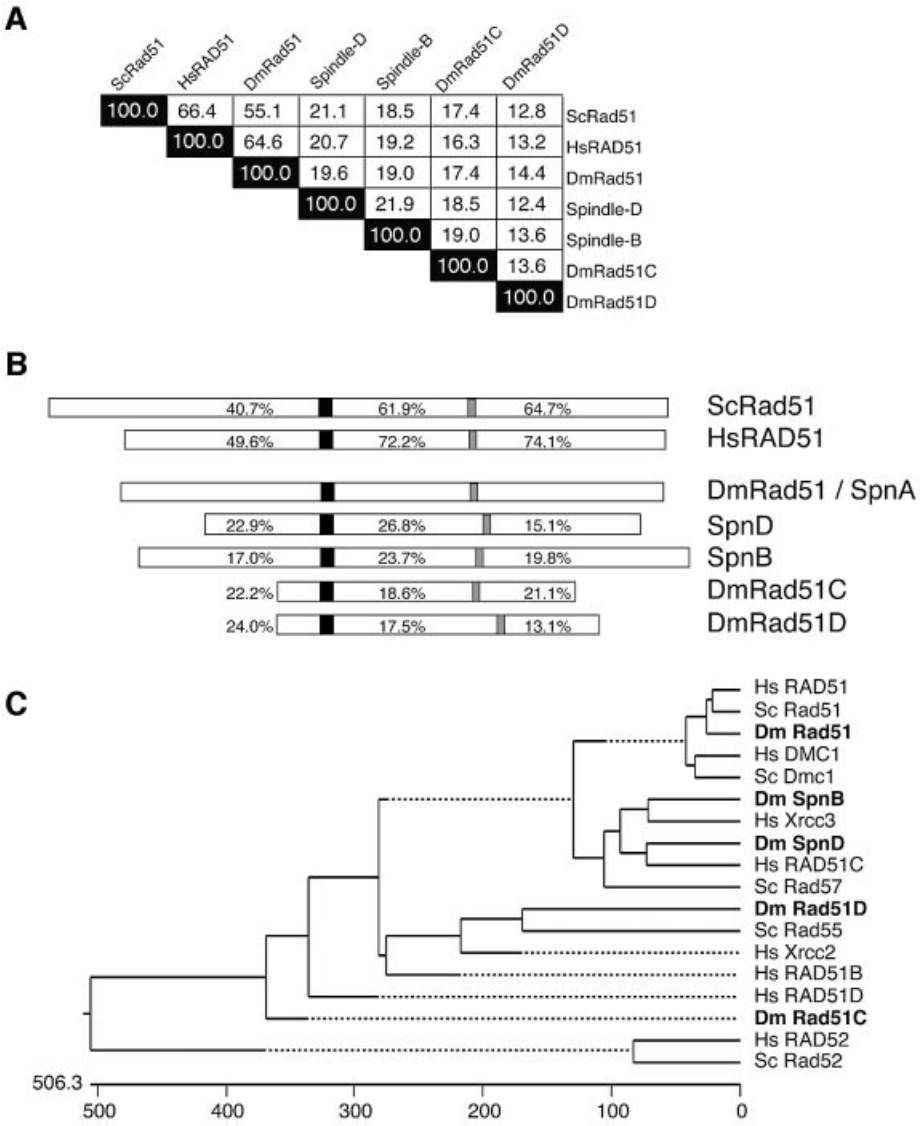


Fig. 3. Sequence comparison of *Drosophila* Rad51 family members with those of yeast and human. A multiple sequence alignment, using complete protein sequences from known *S.cerevisiae*, human and *Drosophila* Rad51 family members, was assembled by the CLUSTAL V method (Higgins and Sharp, 1989; Higgins *et al*., 1992), which is included in the MegAlign module of the Lasergene sequence analysis software suite (DNASTAR, Inc.), and utilizing PAM250 residue weighting. (**A**) Summary of alignment results. Numbers represent percent identity between proteins. (**B**) Each protein is presented as a diagram in actual relative size and aligned with respect to the conserved Walker-A box motif (black). The Walker-B box is indicated by a gray rectangle. The percentages represent the level of identity, within either the N-termini, the central core domains containing both Walker boxes, and the C-termini, with respect to the *Drosophila* Rad51 gene. (**C**) Phylogenetic analysis of eukaryotic Rad51 family members. ScRad52 and HsRad52 represent divergent members of the DNA repair proteins and are shown for comparison.

Drosophila Rad51/SpnA is not necessary for viability

The molecular characterization of all 25 *spnA* alleles revealed 22 missense mutations ($spnA^{009A}$ and $spnA^{032B}$ contained the same mutation) and two stop codon mutations. Each missense mutation affects an amino acid conserved from yeast to human Rad51 (Figure 2A; Table I). Western analysis using an antibody that was raised against the entire DmRad51 protein revealed that $spnA^{093A}$, which has an early stop codon at amino acid 70, produces no detectable protein and classifies as a protein null. The other nonsense allele $spnA^{048B}$ introduces a late stop at amino acid 265 and produces a truncated protein (Figure 2B). We also analyzed three missense alleles, $spnA^{087A}$ and $spnA^{010A}$, which have missense mutations in the Walker-A box and Walker-B box, respectively, and $spnA^{057}$, which has a change within the DmRad51 core domain. Western analysis revealed that the missense alleles produce stable proteins that are of similar size to the wild-type protein (Figure 2B). Since all alleles of *spnA* including the null allele $spnA^{093A}$ were viable *in trans* to the deficiency, *Df(3R)X3F*, we conclude that SpnA function is required for oogenesis but is not essential for normal cell viability.

Drosophila has five Rad51 family members

DmRad51/spnA is predicted to encode a 336 amino acid protein. It shows 55.1% identity to *S.cerevisiae* Rad51 over the entire protein (Figure 3A; McKee *et al*., 1996). Even higher conservation is observed in sequence align-

ment with the mouse and human Rad51 (64.6% identical and ~80% similar) (Figure 3A; McKee *et al*., 1996; Sekelsky *et al*., 2000). There were four members of the *Drosophila* Rad51 family identified from the Genome Project, *rad51-like* (CG7948; *spnA*), *spnB* (CG3325), *rad51C* (CG2412) and *rad51D* (CG6318) (Sekelsky *et al*., 2000) and a Rad51C-like protein (CG31069), which was shown to be encoded by *spindle-D* (Figure 3A–C; Abdu *et al*., 2003). Alignment comparison of all five *Drosophila* Rad51 family members reveals that SpnA protein is the most similar to yeast and human Rad51 (Figure 3A and C). This similarity is not only restricted to the RecA core domain (72.2% identical to HsRAD51) but extends to the N-terminus (49.6%) and C-terminus (74.1%) (Figure 3B). Phylogenetic analysis shows that the other *Drosophila* members, SpnB, SpnD, Rad51C and Rad51D, are more similar to the Rad51 accessory proteins HsXRCC3, HsRAD51C, HsRAD51D and ScRad55 (or HsXRCC2), respectively (Figure 3C; Sekelsky *et al*., 2000; Abdu *et al*., 2003). Based on genome annotation and sequence similarity, we conclude that Spindle-A is the structural homolog of the yeast and mammalian Rad51 protein.

spnA is expressed in both the germline and the soma

Germline specific expression and the sterility of DMC1-deficient mice defined DMC1 as a meiosis-specific component of the homologous recombination complex (Habu *et al*., 1996; Pittman *et al*., 1998). However, genome-wide search failed to identify a clear Dmc1 homolog in *Drosophila*. Considering that a Dmc1-like gene would be expressed exclusively in the germline, we examined whether DmRad51/SpnA or any of the other *Drosophila* Rad51 family members are meiosis specific by determining germline and soma gene expression of the five *Drosophila* Rad51 genes by RT–PCR (Figure 4A and B). Rad51 gene expression profiles from wild-type females, flies from *tudor* mutant females, which lack germline (Boswell and Mahowald, 1985), and to males, which fail to undergo meiotic recombination, were compared. For each Rad51 gene, primers were designed to specifically amplify a fragment of the corresponding transcript. As a control for germline-specific expression, we also analyzed *oskar* and *nanos* RNA by RT–PCR (Ephrussi *et al*., 1991; Wang and Lehmann, 1991). *spnA*, *spnB* and *rad51C* RNA are expressed in both males and females (Figure 4A and C). Furthermore, their expression is not limited to the female germline (Figure 4B). Interestingly, *rad51D* and *spnD* RNA appear to be expressed almost exclusively in the germline of adult females. Thus, *Drosophila* may have two Rad51 family members that are specifically involved in meiotic recombination and functionally equivalent to Dmc1.

spnA mutants are sensitive to ionizing radiation

Since *spnA* and *spnB* are expressed in the soma as well as the germline, we wished to investigate a possible somatic function for these genes. To determine if these proteins function in the mitotically active cells of the soma, *spnA*, *spnB* and *spnB spnA* doubly mutant embryos and larvae were exposed to 20 Gy of ionizing radiation (X-rays) and examined for survival. The progeny of heterozygous

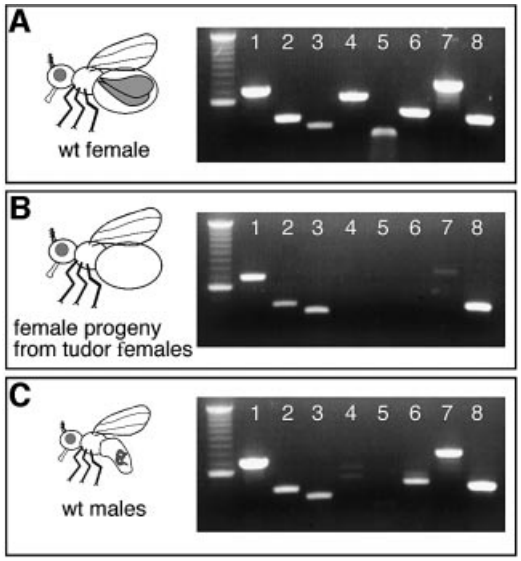


Fig. 4. Gene expression profiles of *Drosophila* Rad51 family members from wild-type females, wild-type males, and germline-depleted females. RT–PCR was performed with total RNA from: (**A**) wild-type females (*spnA/TM3*); (**B**) female progeny of *tud/Df* mutant females, which lack germline tissue; and (**C**) wild-type males. 1, d*mRad51* (687 bp); 2, *spnB* (436 bp); 3, *dmRad51C* (380 bp); 4, *dmRad51D* (620 bp); 5, *spnD* (302 bp); 6, *nanos* (533 bp); 7, *oskar* (760 bp); and 8, *rp49* (430 bp). *nanos* and *oskar* represented germline controls. The residual *oskar* RNA expression in the germline-depleted flies may relate to its reported expression in the nervous system (Dubnau *et al*., 2003). The first lane in each gel is a 100 bp DNA ladder (Roche).

parents were irradiated at either 0–24 h, 24–48 h or 48–72 h after egg laying (AEL). The survival of the irradiated progeny was compared to that of their unirradiated siblings and a survival ratio was established (see Materials and methods). At the irradiation dosage chosen, there was no apparent difference in the survival of irradiated and unirradiated control flies (survival ratio close to 1) for all irradiation times, indicating an insensitivity to this dose of irradiation (Figure 5A and B). At the same dose, *spnA*, *spnB* and *spnB spnA* doubly mutant embryos and larvae show an age-dependent sensitivity to ionizing radiation (Figure 5A and B). When irradiated during embryogenesis (0–24 h AEL), there was little difference in survival between mutant and control. However, during later larval stages (48–72 h AEL) there was a significant difference between the survival of *spnA* mutant larvae and their heterozygous control siblings (Figure 5A). This increase in sensitivity to irradiation with age is likely due to maternal proteins present in the developing embryos; as the animals get older less maternal protein is present due to degradation. Heterozygous control progeny experience the same degradation of maternal product but survive due to their ability to synthesize necessary gene product *de novo*. While *spnA* mutants show a striking sensitivity to irradiation at late third instar (48–72 h AEL), *spnB* mutants show only a modest sensitivity to ionizing radiation (Figure 5B). This is in contrast to the insensitivity observed when *spnB* mutants were exposed to MMS (Ghabrial *et al*., 1998). *spnB spnA* double mutants do not show a synergistic sensitivity to ionizing radiation (Figure 5A and B), rather, they behave similar to *spnA*

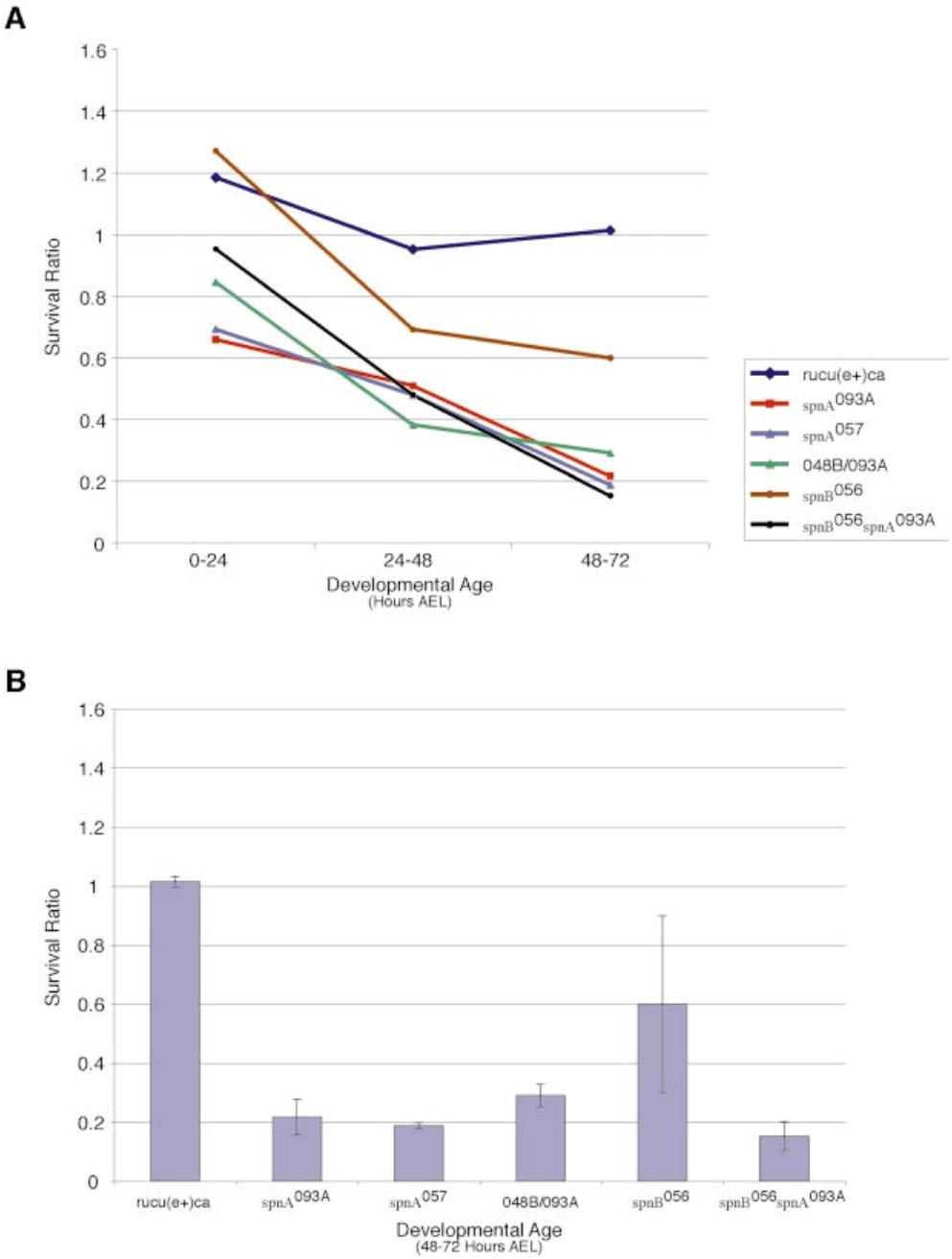


Fig. 5. X-ray sensitivity of *spnA*, *spnB* and s*pnBspnA* mutants by developmental age. (**A**) Survival ratios of 0–24 h AEL embryos, 24–48 h AEL larvae, and 48–72 h AEL larvae after 20 Gy of ionizing radiation. (**B**) Survival ratios of the 48–72 h AEL third instar larval age. The average sensitivity of four independent experiments is reported with standard deviation.

alone, suggesting that the two genes are part of the same non-redundant pathway. Most importantly, our results show that SpnA does indeed play a role in the soma to protect against chromosomal damage inflicted by DNA damaging agents.

dmRad51/spindle-A mutants are defective in female meiosis

To better characterize the role of SpnA during oogenesis, we analyzed in detail the meiotic defect of the *spnA* null allele. We asked whether meiotic chromosome synapsis is affected in the mutants, whether DNA breaks occur during meiosis and can be repaired in the mutant, and finally whether *spnA* mutants indeed cause activation of a meiotic checkpoint. In *Drosophila*, each germline stem cell, at the anterior tip of each ovariole, divides asymmetrically to produce a new stem cell and a differentiating cystoblast (Figure 6A; Spradling, 1993). The cystoblast undergoes four rounds of mitotic division with incomplete cytokinesis to generate a cyst of 16 cells. The cells within a cyst remain interconnected by cytoplasmic bridges called ring canals. The initiation of meiosis is indicated by the appearance of the synaptonemal complex (SC), which assembles in region 2a of the germarium in the four cells of the cyst that form first and thus contain either three- or four-ring canals. The two four-ring canal cells will become

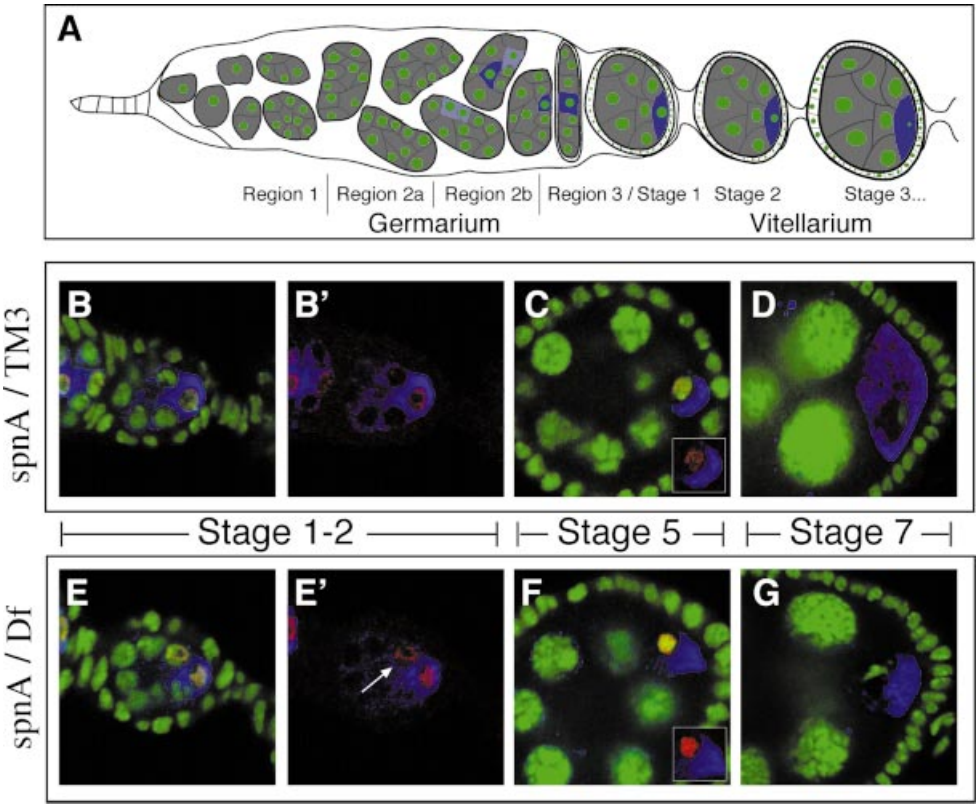


Fig. 6. Synaptonemal complex formation and resolution during wild-type and *spnA* oogenesis. (**A**) Diagram of the anterior of a *Drosophila* ovary. Germline cells are in gray. The DNA is in green and blue represents expression of the oocyte-specific factor, Orb (Christerson and McKearin, 1994), with light blue representing early low levels and darker blue representing full, localized expression within the oocyte. (**B–D**) Confocal images of different oogenesis stages from a wild-type (*spnA*^{093A}/*TM3*) ovary. (**E–G**) Confocal images of similar oogenesis stages from a *spnA* [*spnA*^{093A}/*Df(3R)X3F*] ovary. DNA is stained with OliGreen (green). Oocytes are marked by immunostaining for Orb (blue). SC is made visible by anti-C(3)G immunofluorescence (red). (B′) is the same as (B) minus the green channel. (E′) is the same as (E) minus the green channel, with an arrow indicating the losing pro-oocyte. Insets shows oocyte nucleus minus the green channel. All images are single confocal sections.

the pro-oocytes as defined by the persistence of the SC and their accumulation of oocyte specific markers in region 2b (Page and Hawley, 2001). By stage 1 of oogenesis, the SC, as observed by immunostaining for the *Drosophila* SC component C(3)G, and oocyte markers are restricted to a single cell, the future oocyte. As the egg matures, C(3)G begins to lose association with chromatin and the SC is no longer observed.

We followed the distribution of C(3)G in *spnA* mutant germline cysts. As in the wild type, C(3)G expression is first detected in region 2A (data not shown). However, C(3)G restriction to the oocyte and its dissolution from the chromatin are delayed. At stage 1 of oogenesis, while the SC is always restricted to just the oocyte in wild type, it persists from time to time in both pro-oocytes in *spnA* mutants, similar to what was observed previously. (Figure 6B, B′, E and E′; Huynh and St Johnston, 2000). Furthermore, when C(3)G staining decreases in the maturing stage 5 oocyte, it remains in the mutant (Figure 6C and F). By stage 7, the staining is no longer detected in the oocyte of either wild-type or *spnA* mutant egg chambers (Figure 6D and G). These results suggest that synapse formation is appropriately initiated in *spnA* mutants but the failure to repair broken DNA causes a delay in the resolution of synapsis, first in the cyst that will not become the oocyte and subsequently in the oocyte as it progresses through meiosis.

DSBs are not processed efficiently in spnA oocytes

The meiotic phenotype of *spnA* suggests that there may be a delay in proper meiotic chromosome dynamics due to the failure to repair DSBs. To visualize DSBs cytologically, we used an antibody that recognizes γ-H2AX, a phospho-epitope of the human histone H2A variant, H2AX, which becomes phosphorylated upon DSB formation (Redon *et al*., 2002). The phospho-epitope is conserved in *Drosophila* histone variant HIS2AV and becomes phosphorylated in the event of DSBs, whether induced exogenously or during meiosis (Madigan *et al*., 2002; Jang *et al*., 2003). During wild-type meiosis, we observed few γ-HIS2AV foci, presumably due to the rapid repair of DSBs and the formation of viable recombination intermediates. When observed, γ-HIS2AV foci were found in only one cell in region 2a of the germarium (Figure 7A and A′). This early appearance of γ-HIS2AV, before the restriction of other oocyte markers to a single cell, suggests that the regulation of DSB formation and persistence may be a critical event in oocyte specification. γ-HIS2AV foci were not observed from region 2B onwards (Figure 7B). Thus, DSBs are rapidly processed and recombination intermediates are formed concomitant with oocyte specification. In contrast, *spnA* mutant germaria show a more robust HIS2AV activation in one or two cells of a growing cyst in region 2a (Figure 7C and C′), suggesting that in *spnA* DSBs form at the normal time

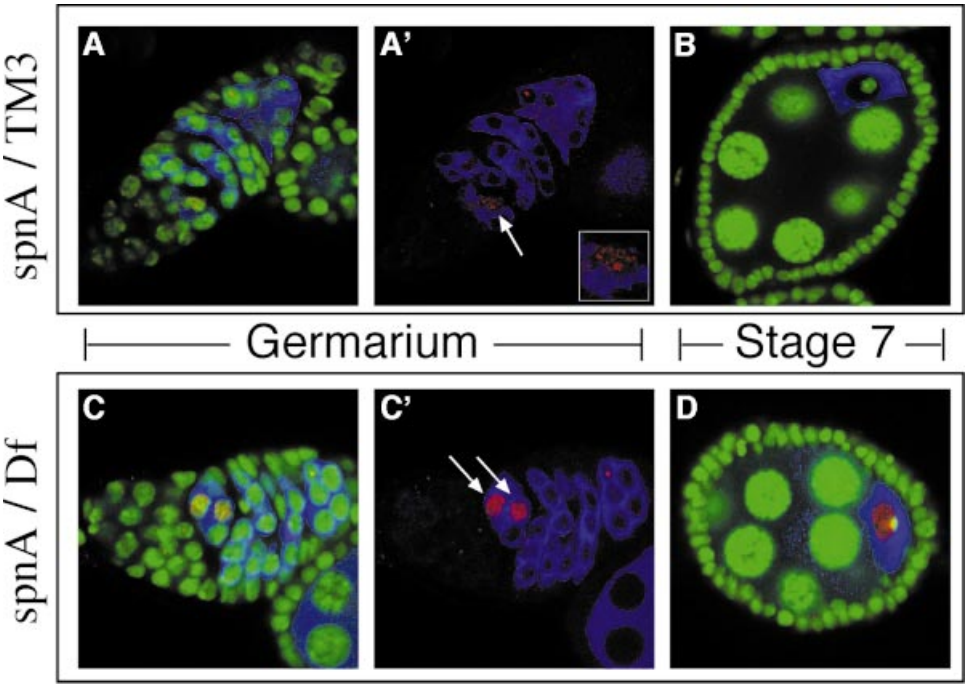


Fig. 7. Persistence of γ-HIS2AV staining suggests a defect in the repair of meiotically induced DSBs. (**A** and **B**) Confocal images of different oogenesis stages from a wild-type (*spnA*^{093A}/*TM3*) ovary. (**C** and **D**) Confocal images of similar oogenesis stages from a *spnA* [*spnA*^{093A}/*Df(3R)X3F*] ovary. DNA is stained with OliGreen® (green). Early cysts and the oocytes are marked by immunostaining for Orb protein (blue). (A and A′) γ-HIS2AV speckles the DNA in one cell (A′, red, arrow), presumably the oocyte. A 2× zoom of that nucleus is provided in the A′ inset. (C and C′) γ-HIS2AV observed in the germarium of *spnA* ovaries. (C′, arrows) Two cells of a common cyst (D) γ-HIS2AV in *spnA* mutants beyond the germarium in the vitellogenic stages of oogenesis. All images are single confocal sections.

but their resolution is delayed. Furthermore, γ-HIS2AV localization is more extensive along the DNA rather than in distinct foci as observed in the wild type, possibly due to the accumulation of unresolved breaks along the chromosomes. HIS2AV activation persists in the oocyte nucleus through later stages of oogenesis suggesting a failure to properly repair DNA breaks (Figure 7D).

The spnA oogenesis phenotypes are linked to defects in DSB repair

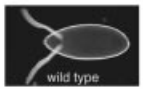
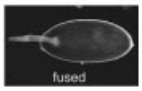
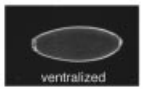
If all the defects observed in *spnA* mutants were due to the activation of a checkpoint upon failure to repair DSBs, one would predict that mutations, which prevented break formation in the first place, would suppress the *spnA* phenotype. This rationale was suggested by results in yeast where mutations in *spo11* suppressed the meiotic sporulation defects of *dmc1* mutations (Roeder, 1997; Bishop *et al*., 1999). Subsequently, it was shown that the eggshell phenotype of two *Drosophila* Rad51 family members, *spnB* and *spnD*, as well as the Rad54 homolog, *okra*, was suppressed in the absence of *mei-W68*, the *Drosophila* homolog of Spo11 (Ghabrial *et al*., 1998). We therefore generated double mutants between *mei-W68* and *spnA* and examined their ability to produce properly patterned eggs. Control females that were efficient at producing DSBs during meiosis [*mei-W68*/+; *spnA*^{093A}/*Df(3R)X3F*] but were defective in SpnA function, produced progeny with *spindle* eggshells (Table IIA). In contrast, in flies defective in DSB production and SpnA function [*mei-W68*/*mei-W68*; *spnA*^{093A}/*Df(3R)X3F*], the *spindle* phenotype was rarely observed (<1%). Furthermore, the oocyte nuclear morphology and Gurken protein localization and distribution appeared normal in the double mutants (data not shown). *mei-W68* also suppressed the embryonic lethality associated with loss of maternal SpnA. In this situation, embryos from doubly mutant females survived to adulthood with a frequency similar to that observed in *mei-W68* progeny alone (data not shown). The fact that all phenotypes associated with *spnA* mutants are suppressed by *mei-W68* suggests that it is indeed the role of SpnA in repair of meiotic-induced DSBs that is essential for normal oogenesis and survival.

The spnA oogenesis phenotype results from the activation of a cell cycle checkpoint

Our data show that DSBs are readily detectable by γ-HIS2AV staining and persist during oogenesis in *spnA* mutants. Unrepaired DSBs or unresolved recombination intermediates lead to the activation of an ATM/ATR-dependent cell cycle checkpoint in mitosis and meiosis, which often causes delays in cell cycle progression in order to repair DNA damage (Roeder and Bailis, 2000). To test whether unrepaired DSBs or unresolved recombination intermediates in *spnA* mutants trigger a cell cycle checkpoint that leads to defects in oocyte development, we wished to inactivate the checkpoint response.

Two genes have been implicated in checkpoint function, the *Drosophila* ATR homolog Mei-41 and the Chk2 homolog DmChk2/Mnk/Loki (Sibon *et al*., 1999; Xu *et al*., 2001). Since *Drosophila mei-41* mutants also show a defect in meiotic recombination (Baker and Carpenter, 1972; Carpenter, 1979), it is difficult to assess the exact step in meiosis that is affected in this mutant. We therefore focused on the checkpoint protein, DmChk2. On its own,

Table II. Suppression of spnA eggshell phenotypes by mei-W68 and chk2

	Maternal Genotype	 wild type	 fused	 ventralized	n
A	$\frac{+}{+}$; $\frac{spnA^{093A}}{Df(3R)X3F,e}$	31%	69%	0%	936
	$\frac{mei\text{-}W68^{1}}{CyO}$; $\frac{spnA^{093A}}{Df(3R)X3F,e}$	25%	70%	5%	958
	$\frac{mei\text{-}W68^{1}}{mei\text{-}W68^{1}}$; $\frac{spnA^{093A}}{Df(3R)X3F,e}$	99%	<1%	0%	1209
B	$\frac{+}{+}$; $\frac{spnA^{057}}{spnA^{093A}}$	6%	87%	7%	840
	$\frac{chk2\ Df^{1}*}{CyO}$; $\frac{spnA^{057}}{spnA^{093A}}$	78%	21%	<1%	1166
	$\frac{chk2\ Df^{1}}{chk2\ Df^{2}}$; $\frac{spnA^{057}}{spnA^{093A}}$	100%	0%	0%	1213

*See Materials and methods for description of chk2 mutant lines.

chk2 mutants do not appear to have a meiotic phenotype (Masrouha *et al*., 2003). As detailed in Table IIB, females doubly mutant for *chk2* and *spnA* produced progeny with wild-type egg shape (100 versus 6%), even a reduction in the copy number of *chk2* (*chk2*/+, $spnA^{057}/spnA^{093A}$) partially suppresses the *spindle* phenotype (22 versus 94%). In addition, the karyosome appears normal in the oocytes from females doubly mutant for *chk2* and *spnA* (data not shown), suggesting that the abnormal nuclear morphology observed in *spnA* mutant oocytes is not the result of fragmented DNA, since the DNA breaks should persist in these double mutants. In contrast to the *mei-W68*;*spnA* doubles, deletion of *chk2* did not suppress the maternal-effect embryonic lethality of *spnA*. Thus, we conclude that the *spnA* phenotype results from the activation of a Chk2-dependent meiotic checkpoint.

Discussion

Screens in *Drosophila* have recovered many mutations that cause disruption to normal meiotic chromosome behavior (Baker and Carpenter, 1972; Sandler, 1974; Sekelsky *et al*., 1999; McKim *et al*., 2002). They were identified based on the ability to recognize abnormal events, such as chromosome loss, non-disjunction or a change in recombination frequency. Mutagen sensitivity screens, similar to those performed in yeast, have also been conducted in *Drosophila* to identify genes necessary for DNA repair (Henderson, 1999). As would be expected, some of these mutagen-sensitive mutants showed meiotic defects as well (Baker *et al*., 1976; Baker *et al*., 1978; Green, 1981). Interestingly, none of the Rad52 epistasis genes of *Drosophila* were recovered from these types of screens. Instead, due to downstream effects on D/V patterning through the activation of a meiotic checkpoint, the *spindle* oogenesis phenotype has proven to be an effective assay by which to uncover these genes. Thus far, four members of the Rad52 epistasis group in *Drosophila* have been found through this approach, (this study; Ghabrial *et al*., 1998; Abdu *et al*., 2003).

In *Drosophila*, there are five members of the Rad51 family. Our analysis confirms that Spindle-A is the structural and functional homolog of the yeast and mammalian Rad51 protein. Biochemical analysis of *in vitro* purified DmRad51 has shown that it has strand exchange capabilities (Alexiadis and Kadonaga, 2002). The other Rad51 paralogs show greater sequence homology to Rad51 accessory proteins, which have been shown to promote Rad51 foci formation on DNA. Here we show that both *rad51D* and *spnD*, in the adult, are expressed specifically in the germline. Therefore, we suggest that they are Rad51 accessory proteins involved in meiotic recombination, compensating for a lack of a *Drosophila* Dmc1 homolog. Initial studies on *spnB* revealed a striking similarity to Dmc1, namely its importance in meiotic recombination and its resistance to the effects of MMS (Ghabrial *et al*., 1998). However, we show that *spnB* RNA is expressed in the soma as well as the germline. Moreover, we present evidence that *spnB* mutant larvae are less tolerant than their wild-type siblings to the DNA damaging effects of ionizing radiation. Based on its sequence homology to XRCC3, it is possible that SpnB

functions as a necessary partner for DmRad51 during meiotic recombination and takes on a supporting role in Rad51 stabilization during DSB repair of the soma (Liu *et al*., 1998; Brenneman *et al*., 2002).

SpnA is not essential for viability

Analysis of Rad51 function in vertebrate development has been difficult due to the early embryonic lethality of RAD51–/– mice. Vertebrate cell culture studies have suggested an essential role of RAD51 in the repair of breaks generated during DNA replication (Sonoda *et al*., 1998), thus providing some explanation for the embryonic lethality in mice. Here we show that *Drosophila* Rad51 null animals can survive to adulthood. Therefore, the requirement for Rad51 in the repair of DNA breaks occurring during DNA replication may not be conserved. However, other possibilities exist. First, maternal Rad51 may persist to repair DSBs occurring throughout embryogenesis. However, we show that female flies doubly mutant for *mei-W68* and *spnA* produce embryos that survive to adulthood, suggesting that neither maternal nor zygotic DmRad51 function are essential for viability. Another possibility is that the Rad51 genes may have partially overlapping, redundant functions. However, neither of the other family members shows strong homology to Rad51. Additionally, flies doubly mutant for the *spnA* and its closest relative, *spnB*, are viable (Gonzalez-Reyes *et al*., 1997) and the next closest paralog, *spnD*, is expressed specifically in the germline, though we only tested adult animals. An alternative explanation, and the one we favor, is the existence of an alternative repair pathway that can compensate in the event of homologous recombination failure. Homologous recombination has been considered the major DNA repair pathway in *Drosophila* (Engels *et al*., 1990; Kurkulos *et al*., 1994; Nassif *et al*., 1994). Recent evidence in *Drosophila* has shown that when the homologous recombination pathway is compromised, the error-prone non-homologous end joining (NHEJ) pathway can compensate and prevent a lethal outcome (Adams *et al*., 2003). Therefore, in *Drosophila*, we would predict that an efficient cooperation must exist between the homologous recombination and NHEJ pathways to prevent the lethal effects of DNA DSBs, presumably with homologous recombination being the primary choice and NHEJ playing a backup role.

SpnA is necessary for oogenesis

During meiotic recombination, crossing over between homologous chromosomes guarantees their proper segregation. Defects in the proper formation of recombination intermediates result in the activation of a pachytene, or meiotic recombination, checkpoint (Roeder and Bailis, 2000). In mice, if defects in chromosomal synapsis or meiotic recombination persist, the result is the activation of the pachytene checkpoint and removal of the arrested germ cells most probably by apoptosis (Odorisio *et al*., 1998; Pittman *et al*., 1998; Baudat *et al*., 2000; Romanienko and Camerini-Otero, 2000). In our study, we show that a meiotic recombination checkpoint is activated in response to a loss of SpnA function. *spnA* mutant females do not show an appreciable defect in egg deposition, suggesting that the apoptotic pathway is not activated in response to the meiotic recombination checkpoint. Moreover, the p53 protein, a strong inducer of apoptosis during the mitotic cell cycle, has been shown not to be involved in the *Drosophila* meiotic recombination checkpoint (Abdu *et al*., 2002). Instead, as our data indicate, the unsuccessful processing of meiotic-induced DSBs results in a Chk2-dependent delay of the meiotic cell cycle. Concomitant with this delay, we observe a defect in the EGFR/TGFα signaling pathway, which results in the production of eggs with dorsal/ventral patterning defects. Thus, our results show a coupling between progression through the meiotic cell cycle and oocyte patterning and development. The ATP-dependent helicase Vasa has been implicated in mediating at least two aspects of meiotic checkpoint activation, Gurken translation and karyosome formation. It remains unclear if Vasa is directly activated by the checkpoint transducer kinase Chk2/Mnk and how defects in DSB repair lead to checkpoint activation. The *spindle* eggshell phenotype has proven to be an efficient assay to identify genes that lead to the activation of the meiotic checkpoint, making *Drosophila* an excellent genetic system to identify additional components that regulate the interplay between DNA repair, cell cycle progression and cell differentiation during meiosis and possibly, as our studies suggest, also mitosis.

Materials and methods

Fly stocks

mei-W68[1] was obtained from the Bloomington Drosophila Stock Center. *chk*2 mutants, kindly provided by B.Suter, were used as a transallelic combination of two deficiency lines that uncover the *chk2* gene locus: *w; Df(2L)pr2b, P[w*+*, barren*+*]/CyO* and *w; Df(2L)be408, P[w*+*, CG107278*+*]/CyO* (Masrouha *et al*., 2003). *tudor* mutant adults were the progeny of *tud*[wc8] *bw/Df(2R)Pu*[rP133] *c px sp* females. The genetic markers used are described in Lindsley and Zimm (1992). Flies were raised on standard cornmeal–molasses medium at 25°C. Embryos were collected on agar–apple juice plates.

Eggshell/chorion preparation

Embryos produced from germline clones of the mutagenized lines were washed once in PBS before being placed into a drop of 100% Hoyer's Mounting media (Ashburner, 1989). Darkfield images were adapted using Adobe Photoshop® 7.0 software.

Allele sequencing

Genomic DNA from flies transheterozygous for each allele of *spnA* and a deletion of the *dmRAD51* gene [*Df(3R)X3F*] and from control starting strains (Line *FRT 161-48* for mutants from the 3R maternal screen and *ru cu[e+]ca* for mutants from the *spnA* allele screen) was prepared as described (Ashburner, 1989). Multiple independent PCRs, following standard manufacturer's protocol, were used to amplify the 1.35 kb genomic region of DmRAD51 (CG7948). Sequencing in both the forward and reverse direction, as described in Supplementary figure 2, was performed on an ABI Prism 3700 machine (Rockefeller University DNA Sequencing Resource Center). SeqMan II (DNASTAR, Inc.) and EditView (Applied Biosystems) were used for analysis of sequencing data.

Western blot analyses

Ovarian extracts were prepared as previously described (Gavis and Lehmann, 1994). Polyclonal anti-DmRad51 was generated using the full-length recombinant Rad51 protein containing a His_6 tag at the C-terminus as antigen. The protein was purified from IPTG-induced *Escherichia coli* BL21 cells containing a Rad51/pET30b expression vector by metal chelation chromatography (Novagen), and used to immunize rabbits. The anti-Rad51 antibody was used at a dilution of 1:1000 (PBST + 2.5% dry milk). Horseradish peroxidase-conjugated goat anti-rabbit was used at 1:5000 (PBST + 2.5% dry milk) and signal was obtained using the LumiGLO®[7] Chemiluminescent Substrate System (KPL).

Mutagen sensitivity

Virgin heterozygote males and females of the specified alleles were mated in yeasted vials for 2 days. The progeny of these vials served as untreated controls. After 2 days, the parents were transferred to newly yeasted vials every day for 4 days. The embryos from these vials were then subjected to 20 Gy of ionizing radiation, using a Torrex 150D X-ray irradiator cabinet, either 0–24, 24–48 or 48–72 h AEL. Upon eclosion, the number of homozygous or transheterozygous mutant progeny was compared to the heterozygous progeny (N = number of mutant/number of wild-type [hets]) for both the untreated and treated populations. These numbers were then compared to establish the level of DNA damage sensitivity ($N_{treated}/N_{untreated} = X$). If X equaled ~1, then there was no sensitivity. If $X < 1$, then the mutant embryos exhibited sensitivity to the ionizing radiation.

Ovary fixation and immunofluorescence

Antibody staining was done as described (de Cuevas *et al*., 1996). DNA staining was performed by incubating the samples for 30 min at room temperature in PBS + 0.1% Tween-20 containing OliGreen® (1:5000; Molecular Probes) and 5 µg/ml RNase A followed by rinsing three times and washing in PBST for 30 min.

All antibodies were diluted in PBS + 0.1% Tween-20 + 0.2% BSA and used at the following dilutions: mouse monoclonal anti-Gurken antibodies ID12 and IF12 at 1:5. (Gift from T.Schupbach), mouse monoclonal anti-Orb antibodies 4H8 and 6H4 at 1:5 (Hybriboma Bank), guinea pig anti-C3G serum at 1:500 (kind gift from R.S.Hawley), rabbit serum for polyclonal anti-CP190 antibody Rb188 at 1:500 (gift from W.Whitfield), commercial rabbit anti-phoshoH2A.X (Ser139) at 1:100 (Upstate Biotechnologies, Lake Placid, NY). Secondary antibodies Alexa-488-conjugated anti-mouse IgG (Molecular Probes), Alexa-488-conjugated anti-rabbit IgG (Molecular Probes), Cy3-conjugated anti-guinea pig IgG (Jackson Immunoresearch), Cy5-conjugated anti-rabbit IgG (Jackson Immunoresearch) and Cy3-conjugated anti-mouse IgG (Jackson Immunoresearch) were each used at 1:500. Images were collected on a Leica DM RBE confocal microscope equipped with a Leica PL APO 40×/1.25NA oil objective using the Leica TCS NT program. Images were adapted using Adobe Photoshop® 7.0 software.

RT–PCRs

Total RNA was obtained from 10–15 whole adult animals using Trizol Reagent® (Invitrogen) and following the manufacturer's protocol. RNA samples were treated using DNA-free™ (Ambion). RT–PCR was performed using SuperScript™ One-Step RT–PCR with Platinum® *Taq* (Invitrogen). Control experiments, using Platinum® *Taq* minus RT, were performed to confirm the absence of contaminating genomic DNA. No signal was ever obtained from the RNA preparation. The primers are listed in Supplementary data S4.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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