Drosophila *mus301/spindle-C* Encodes a Helicase With an Essential Role in Double-Strand DNA Break Repair and Meiotic Progression

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ABSTRACT

mus301 was identified independently in two genetic screens, one for mutants hypersensitive to chemical mutagens and another for maternal mutants with eggshell defects. *mus301* is required for the proper specification of the oocyte and for progression through meiosis in the Drosophila ovary. We have cloned *mus301* and show that it is a member of the Mus308 subfamily of ATP-dependent helicases and the closest homolog of human and mouse HEL308. Functional analyses demonstrate that Mus301 is involved in chromosome segregation in meiosis and in the repair of double-strand-DNA breaks in both meiotic and mitotic cells. Most of the oogenesis defects of *mus301* mutants are suppressed by mutants in the checkpoint kinase Mei41 and in MeiW68, the Spo11 homolog that is thought to generate the dsDNA breaks that initiate recombination, indicating that these phenotypes are caused by activation of the DNA damage checkpoint in response to unrepaired Mei-W68-induced dsDNA breaks. However, neither *mei-W68* nor *mei-41* rescue the defects in oocyte specification of *mus301* mutants, suggesting that this helicase has another function in oocyte selection that is independent from its role in meiotic recombination.

CELLS need to transmit an intact genome to ensure proper development, survival, and reproduction. The accurate replication of their genome requires both monitoring of DNA integrity and repairing of damages to DNA. Double-strand breaks (DSBs) in the DNA arise spontaneously during development or can be produced by ionizing radiations or by mechanical stress. The repair of DSBs is essential for genome stability and tumor suppression, as interactions between the ends of different DSBs can give rise to tumorigenic chromosome translocations (ELLIOT and JASIN 2002; ADAMS *et al.* 2003; SHIVJI and VENKITARAMAN 2004). In eukaryotes, checkpoints are in place to monitor the integrity of the DNA and to avoid the propagation of genomic defects. These checkpoints ensure that a subsequent step in the cell cycle is not initiated in the presence of damaged DNA, allowing additional time for the cell to correct the damage and stimulate the activity of highly conserved repair mechanisms. The DNA damage response in normal cells involves a series of signaling events that include sensors, transducers, and effectors. Central components of these checkpoints in mammals are the ATM/ATR family of phosphatidylinositol-3-OH-kinase-like serine/threonine kinases and their identified targets the checkpoint-1 (Chk1) and checkpoint-2 (Chk2) kinases (KURZ and LEES-MILLER 2004). Homologs of these exist in other eukaryotes where they play similar roles.

Recombination normally occurs during prophase I of meiosis and plays a critical role in homolog segregation and in the formation of viable gametes. Current models for meiotic recombination are based on the double-strand break repair model (SZOSTAK *et al.* 1983; BLANTON and SEKELSKY 2004). In budding yeast, recombination starts with the formation of double-strand DNA (dsDNA) breaks catalyzed by the type II DNA topoisomerase *Spo11*, the homolog of *mei-W68* in Drosophila (CAO *et al.* 1990; MCKIM and HAYASHI-HAGIHARA 1998). Since the lack of function of *mei-W68* abolishes meiotic crossing over and gene conversion, it is likely that recombination in the Drosophila ovary is also initiated by the occurrence of DSBs (MCKIM *et al.* 1998). In Drosophila, the repair of these dsDNA breaks is monitored by a meiotic checkpoint that involves the activity of the Mei-41 kinase (ATR homolog) (HARI *et al.* 1995) and of the Drosophila Chk2 homolog *maternal nuclear kinase* (*mnk*) (OISHI *et al.* 1998; ABDU *et al.* 2002). Its activation results in the modification of two effector proteins, Vasa and Wee1. As a consequence, the meiotic cell cycle is regulated and the efficient translation of *gurken* (*grk*) mRNA is prevented (GHABRIAL and SCHÜPBACH 1999; ABDU *et al.* 2002). Since Grk is required for the proper establishment of axial polarity in oogenesis, mutants unable to repair DSBs during recombination lay eggs harboring polarity defects. In addition, oocytes with unrepaired DSBs show an abnormal chromatin condensation in the oocyte nucleus, a phenotype that

most probably reflects an arrest in meiotic prophase I (González-Reyes *et al.* 1997). Interestingly, since both the polarity defects and the abnormal nuclear morphology are rescued in double mutants with *mei-W68* and *mei-41*, progression through meiosis is coupled to specific patterning events during oogenesis (González-Reyes 1999).

Several genes in Drosophila with a known role in progression through meiosis and in early events of oocyte selection and patterning have been identified. Most of the *spindle*-group of genes were initially identified as maternal-effect mutants with altered eggshell morphology (Tearle and Nüsslein-Volhard 1987; Schüpbach and Wieschaus 1989). Members of this group include *spindle (spn)-A, spn-B, spn-C, spn-D,* and *spn-E* and *vasa, maelstrom, aubergine,* and *okra.* In spite of the pattern defects and the abnormal nuclear morphology of *vasa, aubergine,* and *maelstrom* mutants, these genes seem to be involved only indirectly in meiotic progression. *aubergine* and *maelstrom* are required for translational silencing mediated by RNA interference and microRNAs (Wilson *et al.* 1996; Kennerdell *et al.* 2002; Findley *et al.* 2003), whereas *vasa* is a target of the *mei-41*-dependent checkpoint pathway that occurs in response to dsDNA breaks and acts as a translational regulator of several maternally provided mRNAs, including *grk* mRNA (Styhler *et al.* 1998; Tomancak *et al.* 1998; Ghabrial and Schüpbach 1999).

A detailed analysis of the mutant phenotypes of *spn-A, spn-B, spn-C, spn-D,* and *spn-E* demonstrated the involvement of these genes in the four symmetry-breaking steps that lead to the polarization of the two main body axes of Drosophila (González-Reyes *et al.* 1997). The loss of function of the *spindle* genes in the germline produces defects consistent with their role in the selection of the oocyte, the posterior positioning of the oocyte within the egg chamber, and the polarization of the anterior–posterior and dorsal–ventral axes of the follicle (Gillespie and Berg 1995; González-Reyes *et al.* 1997; Ghabrial *et al.* 1998; Abdu *et al.* 2003; Staeva-Vieira *et al.* 2003). The cloning of *okra, spn-A, spn-B,* and *spn-D* established a clear link between these genes and dsDNA break repair. They are all members of the Rad52 epistasis group, a series of genes isolated originally in *Saccharomyces cerevisiae* because of their role in the response to irradiation damage and that were subsequently found to be deficient in meiotic recombination as well (Symington 2002; Richardson *et al.* 2004). *okra* encodes the Drosophila homolog of the yeast DNA-repair protein Rad54, a chromatin-remodeling dsDNA-dependent ATPase with a known function in DSB metabolism; Spn-A is homologous to yeast Rad51, a protein with an essential role in DNA repair and meiotic checkpoint control; *spn-B* is another Drosophila homolog of *Rad51* and has been shown to participate in the repair of meiotic DSBs; finally, *spn-D* encodes a Rad51C-like protein required exclusively during meiosis. Fur-

thermore, since the patterning defects of *okra, spn-A, spn-B,* and *spn-D* mutants can be suppressed by mutations in *mei-W68* and in *mei-41*, the primary defect in these *spn* mutations seems to be a failure to repair DSBs (Ghabrial *et al.* 1998; Abdu *et al.* 2003; Staeva-Vieira *et al.* 2003; Romeijn *et al.* 2005).

Mutations in the *mutagen-sensitive 301* (*mus301*) gene were recovered in a screen for mutants hypersensitive to chemical mutagens (Boyd *et al.* 1981). During the analysis of the contribution of *spn-C* to the repair of DSBs in the female germline, it was found that *spn-C* was allelic to *mus301* (Ghabrial and Schüpbach 1999). (We have adopted the nomenclature proposed for this locus and its alleles by FlyBase; therefore, we have renamed the *spn-C* gene *mus301*.) Since *mus301* mutants are associated with high sensitivity to chemical damage and regulate progression through meiosis via the *mei-41* checkpoint, it is likely that *mus301* participates in the repair of DSBs in mitotic and meiotic cells. Here we report the identification of *mus301* as a member of the Mus308 subfamily of ATP-dependent helicases and demonstrate its involvement in DSB repair. We also show that the activation of the DNA damage checkpoint triggered in *mus301* mutants requires the function of the checkpoint-2 kinase Mnk. Finally, we find that *mus301* has a role in oocyte selection independent of its requirement for *mei-W68*-induced DSB repair and unrelated to the activation of the *mei-41* DNA damage meiotic checkpoint.

MATERIALS AND METHODS

Fly stocks: Following the nomenclature by FlyBase, the *spn-C[094], spn-C[422],* and *spn-C[660]* alleles have been renamed *mus301[094], mus301[422],* and *mus301[660]*, respectively. These alleles had been described as antimorphs because their phenotype in homozygous or *trans*-heterozygous conditions is much stronger than any in *trans* to a deficiency (González-Reyes *et al.* 1997). Throughout the course of this work, it was discovered that the original *mus301[094], mus301[422],* and *mus301[660]* chromosomes carried an enhancer of the *spindle* phenotype that mapped to the right of *mus301*. Cleaned-up *mus301[094], mus301[422],* or *mus301[660]* chromosomes were obtained by recombining the mutations onto a new background and were used in our phenotypic analyses. Since all cleaned-up chromosomes in *trans* to each other or to a deficiency show a similar penetrance of the mutant phenotypes, they all classify as genetic nulls. The *mus301[D1], mus301[D2],* and *mus301[D4]* mutant alleles have been reported elsewhere (Boyd *et al.* 1981). *mus301[2255], mus301[3198],* and *mus301[4875]* are from the Zuker EMS collection (Koundakjian *et al.* 2004; Laurençon *et al.* 2004) and were kindly provided for by A. Laurençon. *spn-D[349], spn-D[150], spn-E[616],* and *spn-E[653]* are described in detail elsewhere (Tearle and Nüsslein-Volhard 1987; González-Reyes *et al.* 1997). *mei-W68[1]* is a strong allele that eliminates meiotic recombination (McKim *et al.* 1998). *mei-41[D3], grp[6A4],* and *mnk[P6]* are amorphic alleles (Fogarty *et al.* 1997; Sibon *et al.* 1999; Brodsky *et al.* 2004). *Df(2R)LL5* is a deficiency for *mei-W68*. *Df(2L)pr65* is a deficiency for *mnk*. *Df(3L)66C-G28* and *Df(3L)ZP3* (66A9-12; 66B5-11, a gift from P. Maróy) are deficiencies that uncover *mus301*.

Rescue construct: An ∼11.2-kb *Sma*I fragment from cosmid 195B2 (European *Drosophila* Genome Project numbering) was cloned into pBluescript and then digested with *Xho*I and religated to give an ∼6.2-kb *Xho*I–*Sma*I fragment in pBluescript. This fragment contained the ∼4.6 kb of CG7972 with ∼700 bp upstream of the transcriptional start site and ∼900 bp downstream of the poly(A) site. This ∼6.2-kb fragment was cloned directionally into a *P*-element transformation vector to test for rescue of *mus301^094^* mutants.

Sequencing of *mus301* mutant alleles: Twelve primer pairs were designed to enable PCR amplification of the gene plus ∼200 bp upstream of the transcriptional start and ∼400 bp after the translational stop. Genomic DNA was prepared from homozygous or hemizygous mutant females and used as template in PCR reactions that, after purification, were sequenced directly using the PCR primers. At least two independent PCR reactions were sequenced for each primer pair and both strands were sequenced for each of the mutations found.

MMS mutagenesis: MMS mutagenesis was performed as described (Ghabrial *et al.* 1998). Sensitivity to MMS was expressed as a fraction of the percentage expected in the treated vial *vs.* the percentage expected in the control:

$$\text{Sensitivity to MMS} = \frac{[(N_{mut/mut}/N_{mut/\text{balancer}}) \times 100]_{\text{MMS}}}{[(N_{mut/mut}/N_{mut/\text{balancer}}) \times 100]_{\text{control}}}.$$

X chromosome nondisjunction: To determine the frequency of *X* chromosome nondisjunction, *yw;+/+* males were crossed to *+/w, mus301/Df* virgin females and F$_1$ larvae were scored for the presence of *y*. If *X* chromosome nondisjunction occurs, exceptional *yellow X*0 larvae will be obtained, having inherited the *X* chromosome from the male. The percentage of nondisjunction was calculated as

$$[4\textbf{X0}/(N + 2\textbf{X0})] \times 100,$$

where *X0* is the number of *y* larvae obtained and *N* is the total number of larvae scored. (The number of *X0* larvae is multiplied by four to account for the *Y0*, the *XXX*, and the *XXY* progeny that are lethal or cannot be scored. Twice the number of *X0* larvae are added to *N* to account for the lethal *Y0* and *XXX* progeny that would not be counted as part of *N*.)

Staining procedures: Antibody, DNA, and rhodamine–phalloidin stainings were performed according to standard procedures. Detailed protocols are available upon request. DNA was counterstained with DAPI (5 mg/ml; Sigma, St. Louis). Antibodies were used at the following concentrations: mouse monoclonal α-Grk (Ghabrial and Schüpbach 1999), 1/10; rabbit α-γHIS2AV, 1/500 (a gift from K. McKim); mouse α-Orb monoclonal antibodies 4H8 and 6H4 (Lantz *et al.* 1994) from the Developmental Studies Hybridoma Bank (University of Iowa), 1/200 each; guinea pig α-C(3)G (Page and Hawley 2001), 1/500; FITC, Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA), 1/200.

RESULTS

***mus301* is required for oocyte selection and progression through meiosis:** The predominant phenotype of *mus301* mutant females is the production of ventralized egg shells, with phenotypes ranging from fused dorsal appendages to fully ventralized eggs with no dorsal appendages (Figure 1B; Table 1 shows a numerical
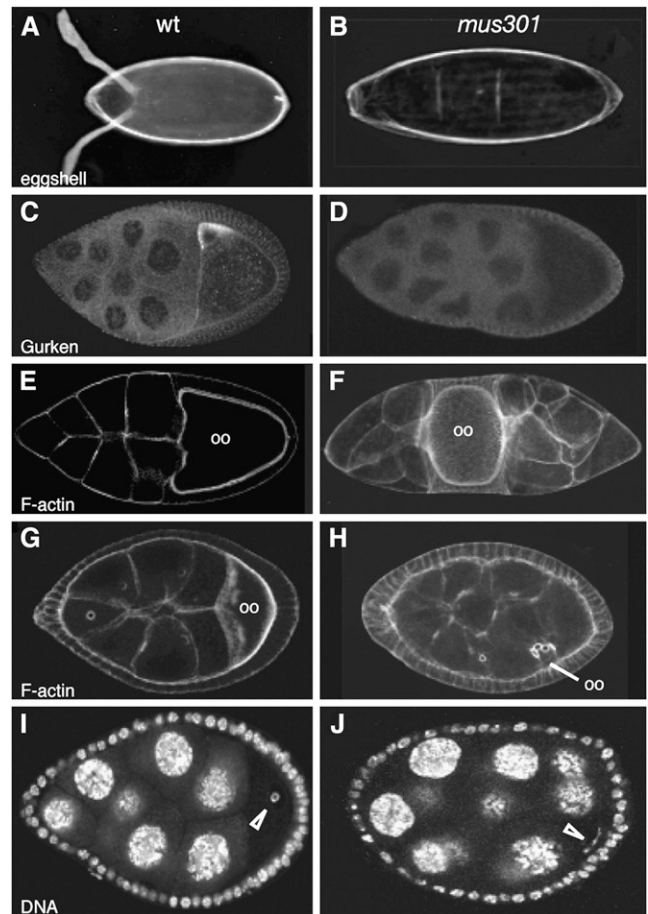

Figure 1.—Phenotypes displayed by *mus301* mutants. (A) Wild-type egg shell. (B) Fully ventralized egg shell. (C) Wild-type S9 egg chamber showing Gurken protein localization. (D) Mutant egg chamber stained and imaged under the same conditions as in C. No Gurken can be detected. (E and G) Wild-type egg chambers stained with rhodamine–phalloidin to visualize the morphology of cells. Both oocytes (oo) are located at the posterior of the egg chamber. (F and H) Mutant egg chambers labeled with rhodamine–phalloidin showing a misplaced oocyte (F) and a small, misplaced oocyte (H). (I) Wild-type follicle with the oocyte's chromatin condensed into a karyosome (open arrowhead). (J) Mutant karyosome. (B and D) *mus301^094^/Df(3L)66C-G28*. (F) *mus301^422^/mus301^660^*. (H) *mus301^D1^/mus301^D2^*. (J) *mus301^660^/Df(3L)66C-G28*.

representation of the mutant phenotypes in Figure 1). This ventralization of the egg shell has been shown to be a consequence of defects in the translation of *grk* mRNA (González-Reyes *et al.* 1997; Ghabrial and Schüpbach 1999). In fact, a significant proportion of mutant *mus301* egg chambers from stage (S) 8 to S10 of oogenesis present a strong reduction or complete absence of Grk protein (Figure 1D). The early and late expression of Grk protein appear to be regulated independently, because early Grk expression is not disrupted in *mus301* mutant females (data not shown). This is also the case in *spn-B* mutants (Ghabrial *et al.* 1998). In a low percentage of *mus301* mutant egg chambers, the

TABLE 1

Phenotypes of *mus301* eggs and egg chambers

Maternal genotype	% egg chambers with a mutant karyosome $(n)^a$	% egg chambers with mutant Gurken levels $(n)^b$	% egg chambers with misplaced oocytes (n)	% ventralized eggs (n)	% egg chambers with small oocytes (n)
w	0 (56)	2 (25)	0 (151)	0 (123)	0 (151)
mus301^094^/Df(3L)66C-G28	100 (161)	ND	2 (296)	49 (553)	ND
mus301^422^/Df(3L)66C-G28	100 (41)	30 (86)	1 (77)	37 (921)	ND
mus301^660^/Df(3L)66C-G28	100 (167)	20 (95)	4 (259)	30 (1251)	4 (123)
mus301^D1^/Df(3L)66C-G28	100 (122)	ND	3 (219)	36 (1784)	ND
mus301^D2^/Df(3L)66C-G28	100 (68)	ND	4 (164)	67 (1209)	ND
mus301^D4^/Df(3L)66C-G28	100 (98)	ND	1 (193)	69 (675)	ND
*mus301^094^/mus301^D1^*	100 (168)	ND	2 (263)	21 (1667)	ND
*mus301^422^/mus301^D1^*	100 (203)	ND	2 (223)	57 (650)	ND
*mus301^660^/mus301^D1^*	98 (167)	ND	0 (198)	45 (1675)	ND
*mus301^D2^/mus301^D4^*	99 (154)	ND	15 (189)	21 (220)	12 (189)

ND, not determined.

^{a} Karyosomes were scored in S3–S7 egg chambers.

^{b} The levels of Gurken protein were found wild type in hemizygous mutant cysts up to S6; the strong reduction or complete absence of Gurken protein was scored in S8–S10 egg chambers.

oocyte is not localized posterior to the nurse cells (Figure 1F), most probably as a consequence of a delay in oocyte selection (GONZÁLEZ-REYES and ST JOHNSTON 1998). In addition, a low proportion of mutant oocytes fail to reach a wild-type size (Figure 1H), indicating that the defect in oocyte selection observed in *mus301* mutants may prevent the normal growth of this cell by affecting the directional transport of cytoplasmic constituents from the nurse cells into the oocyte. The most penetrant phenotype of *mus301* mutant egg chambers is the lack of a karyosome, a solid sphere of compacted chromatin. Nearly all of the mutant oocytes fail to compact their chromatin into a karyosome. Instead, the chromatin fibers of mutant oocytes adopt a fragmented, thread-like appearance (Figure 1J). Since the fragmented karyosome phenotype is rescued by mutations that prevent recombination or the activation of the meiotic DNA damage checkpoint, it has been suggested that *mus301* plays a role in the regulation of meiosis (GHABRIAL and SCHÜPBACH 1999).

The *mus301* gene encodes a new member of the Mus308 subfamily of helicases: *mus301* had been mapped to 66B8-11, a region uncovered by two overlapping deficiencies, *Df(3L)ZP3* and *Df(3L)66C-G28*. The breakpoints of the above deficiencies were mapped onto a contig of P1 clones from the Berkeley *Drosophila* Genome Project using the available sequence-tagged sites (STSs). Next, two P elements inserted in the vicinity of 66B were mapped onto the P1 walk, P{lacW}0903/14 and P{lacW}0898/11 (DEAK *et al.* 1997), in the area deleted by deficiencies *Df(3L)ZP3* and *Df(3L)66C-G28*. Male-mediated recombination (PRESTON and ENGELS 1996) placed *mus301* distal to the P{lacW}0903/14 insertion point and proximal to the *Df(3L)ZP* deficiency breakpoint. We then mapped a cosmid contig from the

European *Drosophila* Genome Project (SIDEN-KIAMOS *et al.* 1990), spanning *mus301* onto the P1 contig using cosmid STSs and thereby defining the area in which *mus301* must lie to a >120-kb region. Meiotic mapping, combined with the use of two restriction fragment length polymorphisms discovered in the region, placed *mus301* within an ~50-kb fragment. One of the open reading frames predicted to lie in this region (CG7972) encodes a polypeptide very similar to the C-terminal domain of *mus308*, a helicase involved in DNA crosslink repair (HARRIS *et al.* 1996). Taking into account the role of *mus301* in DNA repair (GHABRIAL and SCHÜPBACH 1999), we tested the possibility that *mus301* was an allele of CG7972. We first demonstrated that a genomic construct containing CG7972 is able to rescue the egg shell and karyosome phenotypes of the *mus301^094^* allele (not shown). Second, the sequencing of six mutant alleles of *mus301* revealed missense mutations within the coding region of CG7972 (Figure 2 A and Table 2). From these observations we conclude that the CG7972 gene encodes Mus301, a finding in agreement with recent results that identified *mus301* as being allelic to CG7972 (LAURENÇON *et al.* 2004).

mus301 is predicted to encode a 1051-amino-acid (117-kDa) protein with a strong sequence similarity to Drosophila Mus308. Mus308 is a 229-kDa protein involved in the repair of DNA crosslinks whose amino terminal domain contains the seven motifs characteristic of DNA and RNA helicases, and the carboxy terminal domain shares similarity with the polymerase domains of prokaryotic DNA polymerase I-like enzymes (HARRIS *et al.* 1996). Like Mus308, Mus301 contains a series of conserved motifs characteristic of ATP-dependent DNA/RNA helicases belonging to the superfamily 2 (SF2). These regions of Mus301 are mostly distributed
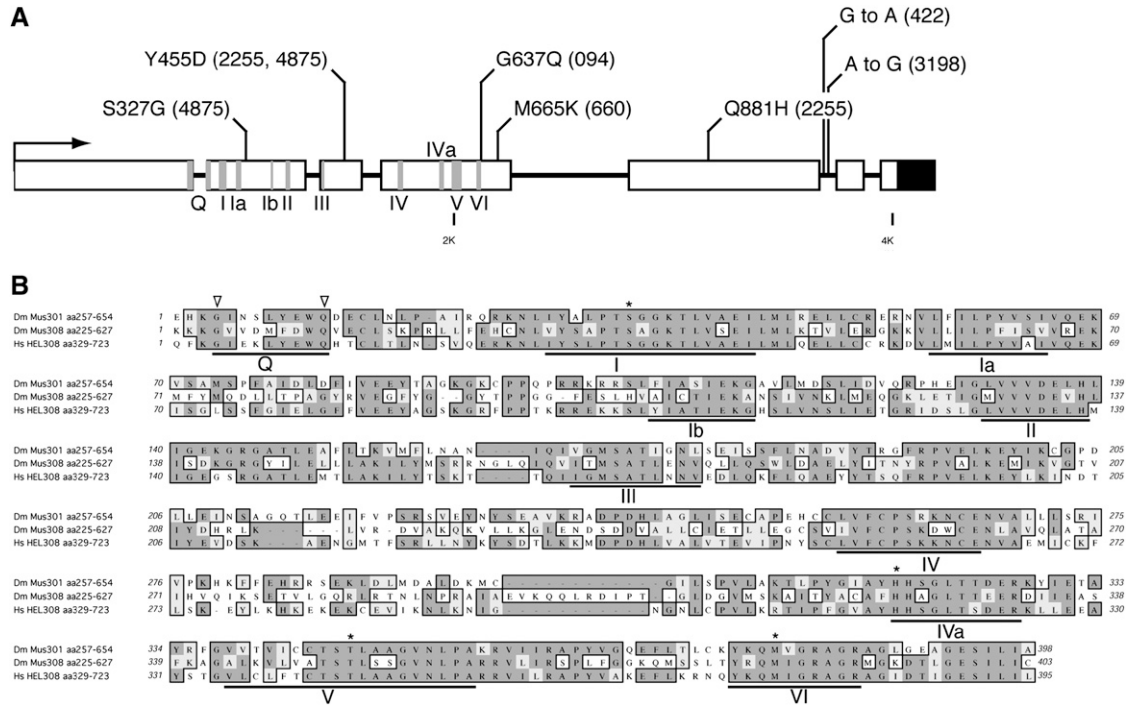
FIGURE 2.—The SF2 helicase Mus301 is a member of the Mus308 subfamily. (A) The genomic structure of *mus301* (CG7972); boxes represent exons; shaded boxes indicate conserved motifs of SF2 helicases. Mus301 possesses nine conserved motifs characteristic of SF2 helicases, and motif IVa, present in the Mus308 subfamily of helicases. The Q motif is split between exons 1 and 2. The rest of the motifs are contained in exons 2, 3, and 4. The molecular lesions identified in six *mus301* mutant alleles are shown. Missense amino acid substitutions or base changes and allele numbers (in parentheses) are indicated. The solid box represents the 3′-UTR. (B) Alignment of the helicase domains of *D. melanogaster* Mus301 and Mus308 and human HEL308. Open arrowheads indicate the conserved G and Q residues of the Q motif; asterisks denote the unique amino acid variations found in the Mus308 subfamily of helicases.

along exons 2, 3, and 4 and include the nine best-conserved motifs in SF2 helicases, the Q box, and domains I, Ia, Ib, II, III, IV, V, and VI. In addition, Mus301 also contains both the IVa motif—an amino acid stretch present in the Mus308 subfamily—and a subset of invariant residues that characterize the Mus308 subfamily but that are unusual among other DEAD-box helicases

(Figure 2) (GORBALENYA *et al.* 1989; HARRIS *et al.* 1996; TANNER *et al.* 2003; TUTEJA and TUTEJA 2004). Thus, Mus301 is a new member of the Mus308 subfamily of SF2 helicases.

The analysis of the molecular lesions in several *mus301* mutant alleles is shown in Figure 2 and Table 2. The *mus301^094* allele is a missense mutation in the highly conserved G637 of motif VI and behaves as a genetic null. Like *mus301^094*, the *mus301^660*, *mus301^2255*, and *mus301^4875* alleles map to exons and affect positions conserved in *Drosophila melanogaster* Mus308 and in human, mouse, and *Caenorhabditis elegans* homologs of *mus301* (Figure 2B and data not shown). Of the remaining sequenced alleles, *mus301^422* introduces a G-to-A change in the conserved splice donor site +5 of intron 5; *mus301^2255* contains a second missense mutation in exon 5 and *mus301^3198* harbors a base substitution in the fifth intron (Table 2). Since three of the strongest *mus301* mutant alleles, *mus301^094*, *mus301^422*, and *mus301^660*, are viable over a deficiency for the locus, or in *trans*-heterozygous combinations, we conclude that *mus301* is not an essential gene.

Mus301 is the Drosophila ortholog of human HEL308: *Dm* Mus301 is 20% similar and 11% identical at the amino acid level over its full length to *Dm* Mus308.

TABLE 2

Molecular characterization of some *mus301* alleles

Allele no.	Base change (+1 <u>A</u>TG)	aa change	Maps to
094	G2101A	G637Q	Fourth exon
422	G3652A		Fifth intron
660	T2185A	M665K	Fourth exon
2255	T1479G	Y455D	Third exon
	G3143T	Q811H	Fifth exon
3198	A3658G		Fifth intron
4875	A1038G	S237G	Second exon
	T1479G	Y455D	Third exon

The 094, 422, and 660 alleles were isolated in the Tübingen mutagenesis (TEARLE and NÜSSLEIN-VOLHARD 1987); the 2255, 3198, and 4875 alleles are from the Zuker EMS collection (KOUNDAKJIAN *et al.* 2004).
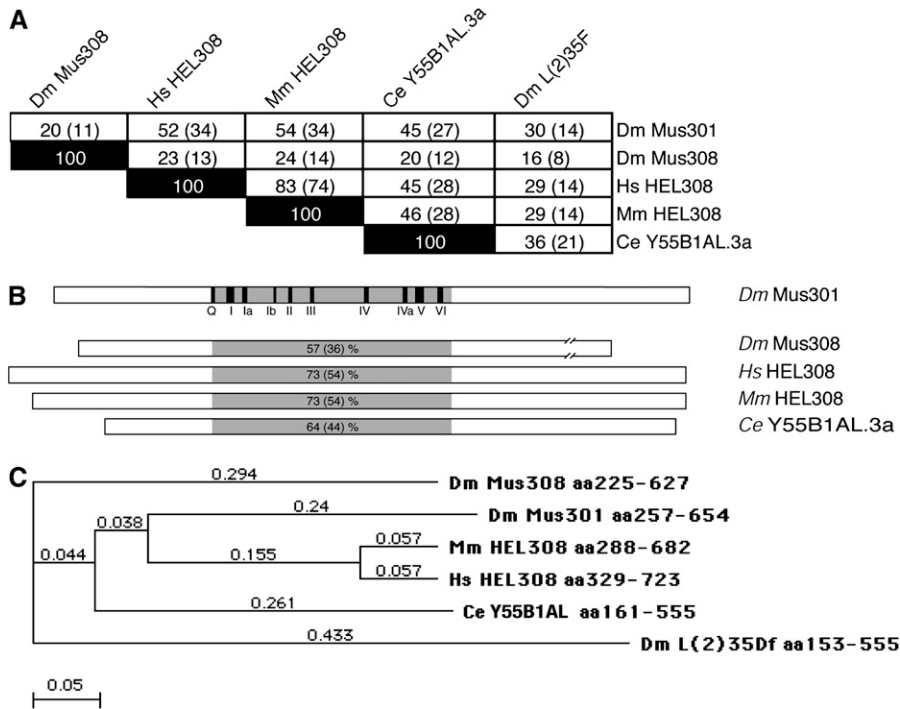
FIGURE 3.—Sequence comparisons of Drosophila Mus301 with other Mus308 subfamily helicases. (A) The complete sequence of *D. melanogaster* Mus301 was compared to that of *Dm* Mus308, human HEL308, mouse HEL308, and *C. elegans* Y55B1AL.3a. The percentages of similarity and identity (in parentheses) are indicated. (B) Percentages of similarity and identity (in parentheses) in the helicase region of *Dm* Mus301 and that of other homologs. Solid boxes represent the SF2 helicase motifs present in *Dm* Mus301. *Dm* Mus308 is not drawn to scale. (C) Phylogenetic analysis of *Dm* Mus301 and related proteins. The tree-building method was "neighbor joining," using the 'best tree' mode; numbers represent uncorrected distances. The Drosophila helicase L(2)35Df is a divergent member of the Mus308 subfamily and is shown for comparison.

This low similarity is due to the fact that *Dm* Mus301 lacks the C-terminal polymerase domain of *Dm* Mus308 and is considerably shorter (1052 aa of Mus301 *vs.* 2059 aa of Mus308). The helicase domains of Mus301 and Mus308 are more highly conserved (57% similar and 36% identical). In an attempt to isolate mammalian proteins involved in DNA crosslink repair, a human and a mouse homolog of Mus308 were identified (MARINI and WOOD 2002). A BLAST search shows that *Homo sapiens* HEL308 and *Mus musculus* HEL308 are more similar to *Dm* Mus301 than to Mus308 (Figure 3, A and B). In fact, the analysis of the phylogenetic tree of the helicase domains of *Dm* Mus301, *Dm* Mus308, *Hs* HEL308, *Mm* HEL308, and *C. elegans* Y55B1AL shows a branched clade in which *Dm* Mus301 and *Ce* Y55B1AL are more closely related to their mammalian counter-parts than *Dm* Mus308 (Figure 3C). On the basis of sequence similarity, we conclude that *Dm* Mus301 is the structural ortholog of HEL308 in mammals.

mus301 is required for chromosome segregation in oogenesis: To assess a possible role for *mus301* in meiosis, we analyzed whether the mutants affect the disjunction of the *X* chromosome. *mus301* hemizygotes were used as they lay some wild-type eggs that give rise to viable larvae. All three mutant alleles analyzed show a high level of *X* chromosome nondisjunction compared to the wild type (*w*) control (Table 3). The percentage of *X* chromosome nondisjunction observed in our experimental conditions is significantly larger than that reported in the initial isolation of the *mus301* alleles (BOYD *et al.* 1981). This difference could be due to the hemizygous conditions utilized in our experiments or to

TABLE 3

X chromosome nondisjunction in *mus301* hemizygous females

Genotype of mothers	Total no. larvae (*N*)	No. of wild-type larvae	No. of *y* larvae (*X0* progeny)	% *X* chromosome nondisjunction
w	3305	3304	1	0.12
mus301[094]/Df	328	313	15	17
mus301[422]/Df	1600	1495	105	23
mus301[660]/Df	202	176	26	41
mus301[094]/Df(3L)ZP3	303	291	12	15
mus301[422]/Df(3L)ZP3	148	138	10	24
mus301[660]/Df(3L)ZP3	188	163	25	42

Females of a given genotype were crossed to *y* males and the presence of *y* larvae was scored, indicating that *X* chromosome nondisjunction had occurred. To avoid unspecific effects due to genetic background, the experiment was repeated with a second deficiency for *mus301, Df(3L)ZP3*.
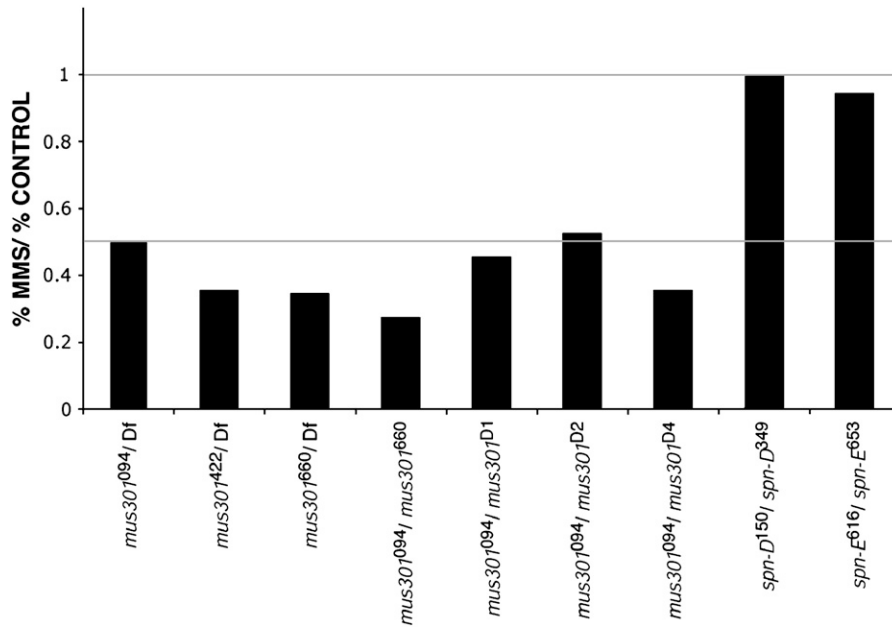
Figure 4.—An essential role for *mus301* in chromosome segregation during meiosis and for DSB repair in mitotic cells. MMS sensitivity of *mus301*, *spindle-D*, and *spindle-E* mutant combinations. MMS sensitivity was expressed as a fraction of the percentage expected in the treated vial *vs.* the percentage expected in the control. *spn-D* and *spn-E* are not required for DSB repair in mitotic cells and are shown for comparison. Df, *Df(3L)66C-G28*.

the fact that previous studies measured nondisjunction of only viable adult progeny, which could introduce a bias for progeny that has disjoined properly. From our observations, we conclude that failure to undergo meiotic recombination in *mus301* mutants (see below) most probably causes a disruption of chromosome segregation during meiosis. Similarly, mutations in *spn-A*, *spn-B*, *spn-D*, and *okra* also impair meiotic recombination and they have been shown to have high levels of *X* chromosome nondisjunction (Ghabrial *et al.* 1998; Staeva-Vieira *et al.* 2003).

mus301 is required for mitotic dsDNA break repair: Given the phenotypic similarities between *mus301* and *okra*, *spn-A*, *spn-B*, and *spn-D* and the involvement of the latter group of genes in the repair of dsDNA breaks, it seems possible that *mus301* also encodes a protein required for DSB repair. To test if this is the case, mutant larvae were assayed for sensitivity to MMS, a mutagen that induces dsDNA breaks. Crosses producing different combinations of *mus301* mutant larvae were fed a solution of 0.8% MMS and the survival of the treated larvae was compared to that of untreated controls. In agreement with the characterization of the original *mus301* alleles (Boyd *et al.* 1981), we found that *mus301* mutants are sensitive to MMS, suggesting that *mus301* is involved in mitotic DSB repair, similar to *spn-A*, *spn-B*, and *okra* (Figure 4). Further confirmation of a role for *mus301* in DSB repair comes from the fact that *mus301* mutants are sensitive to X-rays, which, like MMS, also induce DSBs (Oliveri *et al.* 1990).

mus301 mutants are defective in meiotic dsDNA break repair: During oogenesis, the oocyte is specified in a stepwise manner from a cyst of 16 sibling cells, which are interconnected through actin-rich cytoplasmic bridges called ring canals. Initially, two cells, each

containing four ring canals, behave like "pro-oocytes" as they accumulate oocyte-specific markers. Later on, one of them is selected as the oocyte, while the remaining 15 cells of the cyst acquire a nurse-cell fate. In 16-cell cysts of region 2a of the germarium, the two pro-oocytes and several nurse cells enter meiosis and form synaptonemal complexes (SC), which are proteinaceous structures that connect aligned homologous chromosomes that can be visualized by staining for the SC component C(3)G (Carpenter 1979; Page and Hawley 2001). In region 2b, only the oocyte and an adjacent nurse cell show a distinguishable SC. As the cyst matures, meiosis is restricted to the presumptive oocyte and the SC is found in this cell only in germarial region 3 cysts. By S5–S6 of oogenesis, the SC is disassembled (Figure 5A and data not shown). To determine if the meiotic defects seen in *mus301* mutants are due to a failure in SC formation, we stained mutant germaria with α-C(3)G. As in the wild-type, C(3)G is visible in four cells of region 2a cysts. In contrast to the gradual reduction in the number of C(3)G-positive cells in wild-type cysts, the C(3)G staining persists in several cells per cyst until S2 (Figure 5B and data not shown). Later on, the C(3)G signal is restricted to one cell and disappears at ~S5–S6. These results indicate that the SC forms normally in *mus301* mutant cysts. There is, however, a delay in the restriction of the SC to a single cell, most probably reflecting the late selection of the oocyte characteristic of this mutant. A similar abnormal distribution of the SC was reported for *mus301* mutant cysts using an antibody against an unknown component of the SC (Huynh and St Johnston 2000).

Double-strand breaks in the DNA are produced during meiotic recombination in germline cells of early cysts. One of the earliest known responses to DSB in
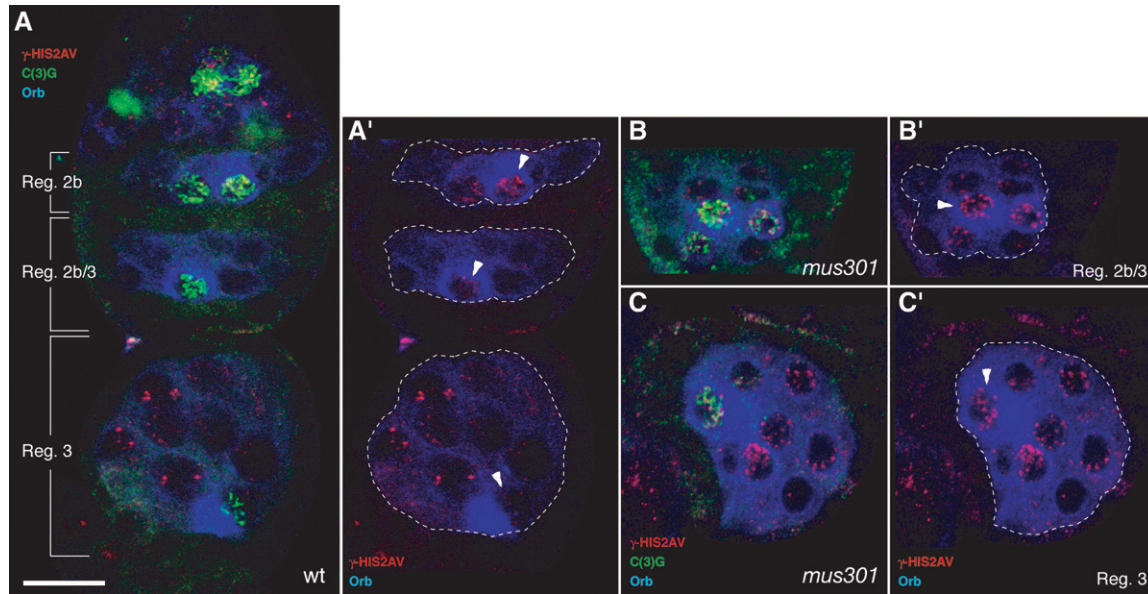
FIGURE 5.—A role for *mus301* in recombinational DSB repair. Wild-type (A) and mutant (B and C) germaria showing the distribution of γ-HIS2AV (red), C(3)G (green), and Orb (blue) proteins. (A) One cyst each from region 2b, region 2b/3, and region 3 is shown (see Figure 6 for a schematic of these germarial stages). γ-HIS2AV foci are abundant in region 2b oocytes, but they are lost in successive stages and are barely detectable in region 3 oocytes. (B and C) γ-HIS2AV foci are visible in region 2b/3 and region 3 mutant oocytes. The sibling nurse cells also possess increased γ-HIS2AV staining compared to wild type. All images are projections of several confocal sections. Dashed lines delineate individual cysts. Arrowheads point toward oocytes. (B and C) *mus301^094^/Df(3L)66C-G28*. Bar, 10 μm.

mammals and *S. cerevisiae* is the phosphorylation of H2A histone variants in the nucleosomes situated in the vicinity of the break. In Drosophila, the phosphorylation of the single histone variant HIS2AV can be detected as soon as 1 min after DSB induction in somatic cells and is removed after 3 hr of exposure to the DSB-inducing agent, thus making this variant histone an excellent marker to study the dynamics of DSB repair (MADIGAN *et al.* 2002). To monitor the presence of DSBs in germline cells of both wild-type and *mus301* mutant cysts, we made use of an antibody that recognizes a phosphorylated form of HIS2AV, γ-HIS2AV. In wild-type female meiosis, γ-HIS2AV staining appears in region 2a of the germarium after the initiation of SC formation. As meiosis proceeds and the DSBs are repaired, the γ-HIS2AV-positive foci disappear, and by germarial region 3 there is no detectable signal in the oocyte (Figure 5A) (JANG *et al.* 2003). Consistent with a role for Mus301 in DSB repair, a large proportion of mutant oocytes show a dramatic accumulation of γ-HIS2AV foci in their nuclei compared to wild-type controls. In addition, these foci persist after region 3 until S3–S4, when they disappear prior to SC disassembly (Figure 5, B and C). A similar increase in the number and persistence of foci can be detected in nurse-cell nuclei. Since the phosphorylation of HIS2AV indicates the presence of DSBs (MADIGAN *et al.* 2002; JANG *et al.* 2003), our observations strongly suggest that DSBs are not processed efficiently in *mus301* mutant oocytes.

***mus301* mutant oocytes activate a Mei-41/Chk2-dependent checkpoint:** The cell cycle and patterning defects observed in *okra, spn-A, spn-B,* and *spn-D* mutants result from the activation of the checkpoint kinase Mei-41 (GHABRIAL and SCHÜPBACH 1999), but there are conflicting data on the identity of the downstream kinases that transduce this signal. On the one hand, it has been shown that Mei-41 acts through the Drosophila Chk2 homolog Mnk1, since *mnk1* mutants suppress the ventralization of *okra, spn-A, spn-B,* and *spn-D* mutants (ABDU *et al.* 2002; STAEVA-VIEIRA *et al.* 2003). On the other hand, MASROUHA *et al.* (2003) have observed that *mnk1* does not suppress the ventralization of *spn-B* or *mus301* mutant alleles. We therefore reexamined this issue by generating *mnk1; mus301* double mutants. Consistent with the results of ABDU *et al.* (2002), we find that double-mutant egg chambers for *mnk* and *mus301* show nearly wild-type levels of Grk protein and a normal karyosome (Table 4), strongly suggesting that, as in the case of *okra, spn-A, spn-B,* and *spn-D, Dm Chk2* is a transducer of the meiotic checkpoint activated in *mus301* mutant oocytes.

Similarly, we analyzed the role of *grapes* (*grp*), a putative serine/threonine kinase with extensive homology to Chk1 kinase in *Schizosaccharomyces pombe* (FOGARTY *et al.* 1997; SIBON *et al.* 1999) in the Mei-41 checkpoint activated in *mus301* mutants. Since *grp; mus301* mutants lay a large proportion of ventralized eggs (<70%, $n = 196$), produce a low frequency of misplaced oocytes (3%, $n = 363$), and fail to form a normal karyosome

TABLE 4

Phenotypes of double-mutant combinations of *mus301* with *grp/DmChk1* or *mnk/DmChk2*

Maternal genotype	% egg chambers with mutant karyosomes (*n*)	% egg chambers with mutant Gurken levels (*n*)
grp[FSA4]	0 (107)	ND
mus301[660]/*Df(3L)66C-G28*	100 (105)	ND
grp[FSA4]; *mus301*[660]/*Df(3L)66C-G28*	100 (193)	ND
mnk[P6]/*CyO*; *mus301*[660]/*TM3*	0 (103)	3 (18)
mnk[P6]/*Df(2L)pr65*; *mus301*[660]/*TM3*	1 (96)	3 (36)
mnk[P6]/*CyO*; *mus301*[660]/*Df(3L)66C-G28*	100 (35)	28 (18)
mnk[P6]/*Df(2L)pr65*; *mus301*[660]/*Df(3L)66C-G28*	4 (23)	7 (14)

Karyosomes were scored in S3–S7 egg chambers. The levels of Gurken protein were scored in S9 and S10 egg chambers. *grp; mus301* double mutants lay ventralized eggs with a similar frequency to *mus301* single mutants (not shown). ND, not determined.

(Table 4), the abnormal patterning and the defective meiosis of *mus301* mutant egg chambers most probably do not involve the activity of *Dm* Chk1. This result adds to the finding that Grp is not the transducer of the pachytene checkpoint in *okra, spn-D,* or *spn-B* mutants (ABDU *et al.* 2002).

mus301 is required for oocyte specification independently of Mei-W68 and of Mei-41 checkpoint activation: During meiosis, DSBs are made to enable recombination to take place. Since a defect in the initial stages of meiosis is likely to occur prior to the defects in pattern formation observed in *mus301* mutant ovaries, we decided to investigate if the latter were a consequence of a failure to proceed through meiosis correctly by analyzing double mutants for *mei-W68* or *mei-41* and *mus301*. In agreement with previous results (GHABRIAL and SCHÜPBACH 1999), we find that *mei-W68; mus301*, and *mei-41; mus301* double mutants possess essentially wild-type karyosomes and Grk protein levels (data not shown). In contrast, the analysis of double mutants shows that the delay in oocyte selection observed in *mus301* mutants is not a consequence of unrepaired DSBs. A fraction of *mus301* mutant cysts accumulate oocyte-specific markers such as Orb protein in the two pro-oocytes until germarial region 3 when compared to wild-type controls (Figure 6). This delay in oocyte selection most probably accounts for the occurrence of misplaced oocytes in older mutant egg chambers (Table 1) (GONZÁLEZ-REYES and ST JOHNSTON 1998). *mei-W68; mus301* mutants behave in this regard like single *mus301* mutants and still show a delay in choosing one pro-oocyte to become the oocyte and display a low percentage of misplaced oocytes (Figure 6; 4% of double-mutant region 2b/3 and region 3 cysts show misplaced oocytes; *n* =135). Similarly, *mei-41; mus301* cysts also present a delay in Orb accumulation in a single cell (Figure 6D). Thus, the delay in oocyte selection characteristic of *mus301* mutants is not a consequence of the activation of the *mei-41* checkpoint. Altogether, our results suggest a role for the Mus301 helicase in oocyte specification independent of the initiation of meiotic recombination by Mei-W68 and the DNA damage checkpoint.

DISCUSSION

Several helicases are involved in the repair of dsDNA breaks. One of the best characterized is the RecBCD complex of eubacteria, which possesses a bipolar helicase activity with a defined role in the homologous recombination repair pathway. The RecBCD recombinase is able to process DNA ends using a combination of helicase and nuclease activities (ANDERSON and KOWALCZYKOWSKI 1997; SINGLETON *et al.* 2004). Also in *Escherichia coli*, RecG helicase has been reported to promote DSB repair subsequent to the activity of RecBCD and RecA (MEDDOWS *et al.* 2004). In eukaryotic cells, defects in DSB repair have been associated with cancer predisposition and genomic instability. For instance, Bloom's syndrome is a rare disorder in humans caused by mutations in the RecQ helicase BLM that results in a predisposition to cancers of all types (HICKSON 2003). Evidence for a role of this helicase in DSB metabolism comes from its involvement in homologous recombination-dependent repair of damaged replication forks (WU and HICKSON 2003) and from the fact that mending of dsDNA breaks in the absence of BLM results in defective products with large deletions (RUNGER and KRAEMER 1989; GAYMES *et al.* 2002). The ortholog of BLM in Drosophila is encoded by *mus309* (KUSANO *et al.* 2001), a gene identified in a screen for hypersensitivity to chemical DNA-damaging agents such as nitrogen mustard, a mutagen that induces interstrand DNA crosslinks, and MMS (BOYD *et al.* 1981). In this screen, a total of 11 complementation groups were isolated, including *mus301* and *mus308*. In contrast to *mus308*, *mus301* is strongly sensitive to both mutagens, implicating Mus301 in the repair of interstrand crosslinks and double-strand breaks. We have identified Mus301 as a new member of the Mus308 subfamily of ATP-dependent helicases and present evidence for a
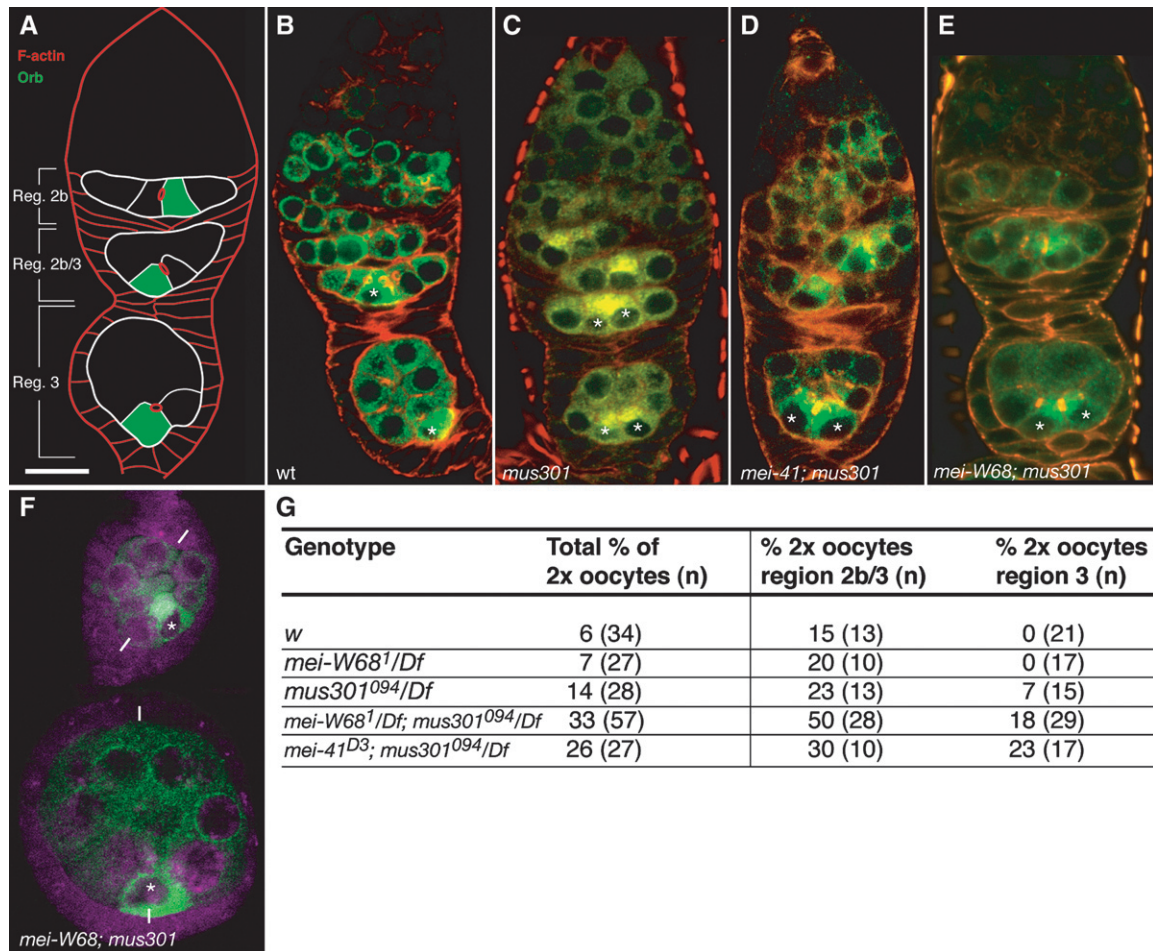
FIGURE 6.—*mei-W68* does not rescue the two-oocyte phenotype of *mus301* mutant cysts. (A) Scheme of a wild-type germarium to show the arrangement of region 2b, region 2b/3, and region 3 cysts. (B–E) Germaria double stained to visualize filamentous actin and Orb protein. (B) Wild-type germarium showing Orb accumulated in a single cell in region 2b and region 3 cysts. (C) Mutant germarium carrying region 2b and region 3 cysts, each containing two cells that accumulate Orb. (D and E) Double-mutant germaria showing region 3 cysts with two cells containing high levels of Orb protein. (F) Double-mutant egg chambers stained with anti-Orb to show a misplaced oocyte. The white bars mark the anterior–posterior axes of the follicles. (G) Germaria of different genetic combinations were analyzed and the number of region 2b/3 and region 3 cysts containing two cells with increased levels of the oocyte marker Orb and each possessing four4 ring -canals (as visualized with rRhodamine—pPhalloidin) were counted as "2×" oocyte" cysts. (B) Wild type. (C) *mus301⁰⁹⁴/Df(3L)66C-G28*. (D) *mei-41ᴰ³/Df(2R)LL5; mus301⁰⁹⁴/Df(3L)66C-G28*. (E and F) *mei-W68¹; mus301⁰⁹⁴/Df(3L)66C-G28*. Asterisks label the cells with highest Orb contents. Bar, 10 μm.

role of Mus301 in the repair of the dsDNA breaks that arise during recombination.

A role for *mus301* in DSB repair and oocyte specification: Recombination begins with the occurrence of dsDNA breaks on one chromatid, catalyzed in budding yeast by the Spo11 protein. Subsequently, the DSB is resected to produce an intermediate with a 3′-overhanging single-strand DNA (ssDNA) tail. Rad51 and Dmc1 proteins then bind to this ssDNA to form a filamentous structure that promotes a search for homologous, nonsister DNA to prime repair DNA synthesis. The requirement of the strand-invasion protein Rad51 in dsDNA break repair is demonstrated by the hypersensitivity to ionizing radiation conferred by mutations in *Rad51* (Symington 2002). The Drosophila genome has five *Rad51* family members (*spn-A, spn-B,* *spn-D,* CG2412, and CG6318) (Staeva-Vieira *et al.* 2003) and mutations in three of these have confirmed their role in the early aspects of DSB repair, as the absence of Spn-A, Spn-B, or Spn-D function leads to the activation of a meiotic checkpoint triggered by unrepaired dsDNA breaks or unresolved recombination intermediates. Furthermore, some of these genes are partially redundant, as double-mutant combinations display stronger phenotypes than single mutants alone (González-Reyes *et al.* 1997). In this context, our finding that, like *spn-A, mus301* mutants do not process DSBs efficiently suggests that Mus301 acts in the same step of DSB repair as Spn-A. Moreover, since *mus301* and several of the *Rad51*-like genes interact genetically, as demonstrated by the enhancement of the mutant phenotypes observed in the double mutants *mus301 spn-A* or

mus301 spn-B (González-Reyes *et al.* 1997), it is likely that *mus301-* and *rad51*-like genes do not act in a linear pathway. Rather, they seem to collaborate in the formation of stable recombination intermediates necessary for efficient DSB repair. In such a scenario, it is interesting to note that *mus301* is the Drosophila ortholog of *Hs* HEL308, a single-stranded DNA-dependent ATPase and DNA helicase of unknown function that *in vitro* is able to translocate on DNA with 3′–5′ polarity and to displace 20- to 40-mer duplex oligonucleotides (Marini and Wood 2002). The considerable sequence similarity (73%) between the helicase domain of Mus301 and that of Hs HEL308 raises the possibility that *mus301* possesses a DNA-unwinding activity with 3′–5′ polarity. In support of our model, budding yeast *mer3* encodes an ATP-dependent DNA helicase that unwinds dsDNA with a 3′–5′ polarity and that stimulates 3′–5′ heteroduplex extension by Rad51 in crossover recombination (Mazina *et al.* 2004). Finally, *okra*, a gene required for the repair of DSB after *P*-element excision and for DNA repair during oogenesis (Kooistra *et al.* 1997; Ghabrial *et al.* 1998; Kooistra *et al.* 1999; Romeijn *et al.* 2005), is the Drosophila homolog of yeast Rad54, a SWI2/SNF2 chromatin-remodeling dsDNA-dependent ATPase that binds Rad51 directly and that stimulates DSB repair in both meiotic and mitotic cells (Mazin *et al.* 2000; Krogh and Symington 2004). Since Okra, Mus301, Spn-A, and, to a lesser extent, Spn-B are also involved in the repair of DNA damage caused by MMS treatment and ionizing radiation in mitotic cells (this work; Oliveri *et al.* 1990; Staeva-Vieira *et al.* 2003), it is likely that the Mus301-Rad51-Okra interaction is also maintained in DSB repair in the soma.

In contrast to the situation in *S. cerevisiae*, the *rad52-* group genes *okra*, *spn-A*, *spn-B*, and *spn-D* were isolated because of their role in egg chamber polarization in oogenesis. Their molecular characterization allowed the establishment of a clear link between DNA repair and pattern formation in the female germline in Drosophila. Additional experiments that involved double-mutant combinations defined the realm of action of the *spindle* genes. They act after the induction of dsDNA breaks by Mei-W68 and are necessary for DSB repair, as demonstrated by the rescue of the meiotic phenotypes of *okra*, *spn-A*, *spn-B*, and *spn-D* mutants in the absence of *mei-W68* or *mei-41*. The phenotypic analysis of *mus301* places this gene at the same level as these *spindle* genes in the recombination pathway. Lack of function of *mus301* prevents the efficient processing of DSBs, thus triggering the activation of the meiotic checkpoint, which in turn induces a Chk2-dependent cell cycle delay similar to the situation in *spn-A*, *spn-B*, and *spn-D* mutants (Abdu *et al.* 2002; Staeva-Vieira *et al.* 2003). In addition, our experiments involving double-mutant combinations for *mei-W68* or *mei-41* and *mus301* have revealed a novel role for *mus301* in oocyte selection independent of initiation of recombination and of DNA damage checkpoint activation during cyst formation. Although the mechanisms by which *mus301* regulates oocyte development independently of *mei-W68* or *mei-41* are unclear, they most probably involve Mus301 helicase activity, since the *mus301* allele used in these experiments carries a missense mutation in a conserved glycine of helicase domain VI and there are not other recognizable domains in the protein. Since there are no detectable meiotic DSBs in the absence of Mei-W68 activity (McKim *et al.* 1998; Jang *et al.* 2003), our observation suggests a new function for Mus301 unrelated to DSB repair in oocyte specification. In this regard, it would be interesting to know if mutations in other genes involved in recombinational DNA repair and in oocyte selection, such as *okra*, *spn-A*, *spn-B*, and *spn-D*, also affect oocyte specification independently of *mei-W68* and *mei-41*.

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