RAD51C deficiency in mice results in early prophase I arrest in males and sister chromatid separation at metaphase II in females

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RAD51C is a member of the RecA/RAD51 protein family, which is known to play an important role in DNA repair by homologous recombination. In mice, it is essential for viability. Therefore, we have generated a hypomorphic allele of Rad51c in addition to a null allele. A subset of mice expressing the hypomorphic allele is infertile. This infertility is caused by sexually dimorphic defects in meiotic recombination, revealing its two distinct functions. Spermatocytes undergo a developmental arrest during the early stages of meiotic prophase I, providing evidence for the role of RAD51C in early stages of RAD51-mediated recombination. In contrast, oocytes can progress normally to metaphase I after superovulation but display precocious separation of sister chromatids, aneuploidy, and broken chromosomes at metaphase II. These defects suggest a possible late role of RAD51C in meiotic recombination. Based on the marked reduction in Holliday junction (HJ) resolution activity in Rad51c-null mouse embryonic fibroblasts, we propose that this late function may be associated with HJ resolution.

Introduction

Bacterial RecA and its yeast orthologue RAD51 are the founding members of the RecA/RAD51 protein family, which plays a crucial role in DNA repair by homologous recombination (for review see Kawabata et al., 2005). After DNA ends are resected at the site of a DNA break, these proteins form a nucleoprotein filament on the single-stranded DNA and catalyze a strand invasion and strand exchange reaction with a homologous region on another DNA molecule to ensure faithful DNA repair. In addition to RAD51, a few other RAD51-like proteins were found in eukaryotes, with their number increasing from three in budding yeast (Rad55, Rad57, and Dmc1) to six in most of the higher eukaryotes (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, and DMC1; Dosanjh et al., 1998). These proteins have 20–30% amino acid sequence similarity and share common functional domains (Miller et al., 2004). The core C-terminal domain contains two functionally important ATP-binding Walker A and B motifs and is linked to a small globular N-terminal domain via a linker region that is important for protein–protein interactions.

Except for DMC1, a meiosis-specific protein with functions overlapping those of RAD51 (Bishop et al., 1992), the other five paralogues are auxiliary to RAD51. In yeast, the purified Rad55/Rad57 heterodimer functions as a mediator of Rad51, enabling it to nucleate on single-stranded DNA in the presence of replication protein A (RPA; Sung, 1997). Chicken DT40
Figure 1. Effect of the hypomorphic allele on RAD51C protein expression and male infertility. (A) Schematic representation of Rad51c alleles. Only the first four exons are shown (Leasure et al., 2001). In the Rad51c<sup>emo</sup> allele, a PGK-neo cassette flanked by FRT (hatched boxes) and loxP (open triangles) sites is inserted into intron 1, and another loxP site is inserted into intron 3. The Rad51c<sup>emo</sup> allele is a product of Cre-mediated recombination at loxP sites, resulting in a deletion of exons 2 and 3. (B) Graph showing the correlation between the Rad51c genotype, fertility status, and testes size. Error bars indicate SD from the mean of the testes size for respective genotypes. Asterisks represent the statistical significance of the differences between the pairs of genotypes indicated by brackets: *, P < 0.05; **, P < 0.001. Examples of tests in relative proportions are shown above the graph. (C) Correlation between the protein level of RAD51C in testes by Western blot analysis and genotype of the mice. (D) Quantitative evaluation of the Western blot shown in C. The histogram represents the amount of RAD51C protein in the testes of mice of various genotypes relative to wild type.

Although all RAD51 paralogues are implicated in RAD51 foci formation, it is unclear how this function is shared between different paralogues and their complexes (Yonetani et al., 2005). RAD51C is part of both BCDX2 and CX3 complexes and, therefore, is believed to play a central role. Being part of two different protein complexes, RAD51C may also have several distinct functions. On one hand, it is involved in the early steps of recombination associated with RAD51. On the other hand, it may also be involved in late steps of homologous recombination, as RAD51C, together with XRCC3, was purified from HeLa cells as part of a small protein complex possessing a Holliday junction (HJ) resolvase activity (Liu et al., 2004). RAD51C is an essential part of this complex because the resolvase activity was lost upon depletion of RAD51C from the fraction or when protein extracts from hamster cells lacking functional Rad51c were tested. However, the role of RAD51C as a resolvase remains controversial. RAD51C lacks an endonuclease domain, and efforts to demonstrate resolvase activity for the recombinant RAD51C protein have not been successful so far. Also, there is no in vivo evidence supporting this late role of RAD51C in recombination.

In this study, we report the generation of a viable mouse model carrying a hypomorphic allele of Rad51c, which enabled us to overcome the early embryonic lethality of a null allele and demonstrate the role of RAD51C in meiotic recombination. We demonstrate that RAD51C is involved in the recruitment of RAD51 at early stages of homologous recombination in spermatocytes. In oocytes, we describe a defect in sister chromatid cohesion at metaphase II. We speculate that RAD51C may also be required at late steps of homologous recombination.

**Results**

**Generation of a hypomorphic allele of Rad51c**

The mouse Rad51c gene consists of eight exons and encodes a 366-amino acid protein (Leasure et al., 2001). Exons 2 and 3...
Aberrant splicing was demonstrated for the reported previously for other genes (Meyers et al., 1998).

To determine the fertility status, 6–8-wk-old male and one female out of 105 mating pairs were infertile. To genotypes are referred to as controls for simplicity), only one 36.6% (sufficient to allow normal growth and development. However, animals were sexually active as indicated by the repeated detection of vaginal plugs, which did not result in pregnancy. In contrast, animals were comprised mostly of multiple layers of round spermatids and usually a single layer of spermatocytes undergoing meiotic divisions (Fig. 2 G). In contrast, most tubules from mutant males contained no mature sperm, few, if any, spermatids, and multiple layers of spermatocytes in the zygotene and pachytene stages of meiotic prophase (Fig. 2 H). Although testes morphology and cell composition improved with age, adult males remained functionally infertile for up to 8 mo of age.

To determine the precise nature of spermatogenesis failure in Rad51cko/neo mice, we analyzed surface spreads of spermatocytes using molecular markers specific for different stages of meiosis. SCP3 and SCP1, which are components of the lateral and central elements of the synaptonemal complex, respectively, were used to identify cells at different stages of meiotic prophase based on the degree of chromosome condensation and interstitial tissues appeared hypertrophied in both infertile (F) and fertile males (H). Arrows in G and H indicate the layers of primary spermatocytes. (E–H) Testes from a 12-wk-old infertile male (E and G) and its infertile littermate (F and H).

**Infertility in males and females**

The residual amount of RAD51C protein in the 142–amino acid region, including a linker and the Walker A ATPase domain. The deletion of these exons resulted in a functionally null allele, Rad51c<sup>neo</sup>, which caused an early postimplantation lethality of Rad51c<sup>neo</sup> mouse embryos (unpublished data). In addition to a null allele, we also generated a hypomorphic gene, Rad51c<sup>hom</sup>, by inserting a neomycin (neo) resistance gene under the control of a phosphoglycerate kinase (PGK) promoter into intron 1 (Fig. 1 A). Disruption of normal splicing by the presence of the PGK-neo cassette has been reported previously for other genes (Meyers et al., 1998). Aberrant splicing was demonstrated for the Rad51<sup>hom</sup> allele by RT-PCR analysis using primers to amplify a region between the first and fourth exons (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200608130/DC1). The sequence analysis predicted that the misspliced transcript was likely to encode for a 76–amino acid polypeptide that included 39 amino acids from the first exon of Rad51c and an additional 37 amino acids from the 3′ region of the PGK-neo cassette. Aberrant splicing resulted in an ~60% reduction in RAD51C protein levels (Fig. 1, C and D). The combination of a hypomorphic and a null allele (Rad51c<sup>hom/neo</sup>) produced mice expressing only 5–30% of the normal level of RAD51C protein (Fig. 1 D).

**Meiotic defects in males**

Tests of infertile Rad51<sup>neo</sup> males were significantly reduced in size (P = 0.042) and weighed 18–48 mg at 8–10 wk of age in contrast to 43–90 mg for fertile Rad51c<sup>neo</sup> males. Furthermore, tests of infertile males also had reduced levels of RAD51C protein compared with that of fertile males (Fig. 1, B and C). Unlike tests of the control males, histological examination of testes from 4-wk-old Rad51c<sup>hom</sup> males revealed seminiferous tubules that were deformed and were often almost devoid of germ cells (Fig. 2, A and B). There was a marked increase in the number of apoptotic spermatocytes in the tests of infertile mice as determined by TUNEL assay (Fig. 2, C and D).

Testis histology of the 12-wk-old infertile Rad51c<sup>hom</sup> males revealed a few tubules containing some mature spermatooza, but the majority still showed abnormal structures compared with control testes (Fig. 2, E–H). Seminiferous tubules from control animals were comprised mostly of multiple layers of round spermatids and usually a single layer of spermatocytes undergoing meiotic divisions (Fig. 2 G). In contrast, most tubules from mutant males contained no mature sperm, few, if any, spermatids, and multiple layers of spermatocytes in the zygotene and pachytene stages of meiotic prophase (Fig. 2 H). Although testes morphology and cell composition improved with age, adult males remained functionally infertile for up to 8 mo of age.
synaptonemal complex formation (Dobson et al., 1994). In control samples, >60% \((n = 184)\) of the spermatocyte population is comprised of cells at late zygotene, when chromosomes are not fully synapsed at their ends (Fig. 3 A), and at pachytene when synapsis is complete (Fig. 3 B). In infertile Rad51cko/neomale mutants, we found that some spermatocytes progressed to the pachytene stage (Fig. 3 D), but 63% displayed reduced numbers of MLH1 foci (Fig. 3 G and Fig. S2, B–E; available at http://www.jcb.org/cgi/content/full/jcb.200608130/DC1), which are markers of the crossover sites (Baker et al., 1996). In contrast, only 11% of the control spermatocytes lacked MLH1 foci on one or more chromosomes, whereas most spermatocytes displayed one or two foci on each chromosome (Fig. 3 E and Fig. S2, A and E). Mutant spermatocytes lacking MLH1 are likely to be at the pre-MLH1 early pachytene stage (Fig. S2 D). Overall in mutant Rad51cko/male, a shift in spermatocyte distribution toward early stages of prophase I was observed (Table I). The number of late zygotene and pachytene spermatocytes was reduced to 40% \((n = 208)\), whereas cells at leptotene (22% in the mutant vs. 7.6% in the control) and at early to midzygotene (30% in the mutant vs. 21% in the control) became the major fractions (Fig. 3 C). The fact that cells at all stages (leptotene-pachytene) of meiosis could be found in these preparations suggests that the phenotype observed in infertile Rad51cko/male males reflects an impairment of spermatogenesis during meiosis I rather than an absolute developmental arrest.

Formation and repair of DNA double strand breaks (DSBs) by homologous recombination ensures a correct pairing and subsequent segregation of homologous chromosomes during the first meiotic division. A successful generation of DSBs and initiation of their repair is indicated by the presence of phosphorylated histone H2AX (γ-H2AX; Fig. 3 J and K) and RPA (Fig. 3 F and H) protein staining in mutant and control spermatocytes at leptotene and zygote (Plug et al., 1997; Mahadevaiah et al., 2001). At pachytene, normally only the sex chromosomes stain positively for γ-H2AX (Fig. 3 J; Mahadevaiah et al., 2001). However, in mutants, more than half of all spermatocytes displayed multiple γ-H2AX foci at pachytene, indicating the persistence of DSBs at this stage (Fig. 3 L). We found no marked difference in the number of RPA foci in mutant and control spermatocytes at various stages of prophase I (Fig. 3 F and H; and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608130/DC1). As RAD51C has been implicated in RAD51 foci formation in mitotic cells, we tested whether RAD51 foci formation was defective in spermatocytes of infertile males. RAD51 plays an important role in the initial steps of homologous recombination by mediating homologous pairing and strand exchange and is normally observed in multiple foci first appearing at leptotene and sharply decreasing at pachytene (Fig. S4, A–C; Ashley et al., 1995). We found that the number of RAD51 foci was reduced about threefold at leptotene and zygote (46–57 foci per cell in the mutant compared with 140–169 foci in the Rad51cko littermate control; Fig. 3, M and O; and Fig. S4, D–G), indicating an early defect in the homologous recombination process.
γ-H2AX staining marks not only unrepaired DNA but also unsynapsed regions along homologous chromosomes. Although some mutant spermatocytes showed only a few γ-H2AX foci on autosomal chromosomes, we found others that had entire chromosomes staining positively for γ-H2AX at pachytene (Fig. 4 A, arrowheads). Such chromosomes also appeared thinner than others when stained for SCP3 (Fig. 4 B, arrowheads) and lacked SCP1 staining (Fig. 3 D, arrow), suggesting that these were completely unpaired autosomal univalents. Abnormal synapsis between homologous chromosomes was further confirmed by karyotyping spermatocytes at metaphase I. We examined metaphase I spermatocytes from 3-wk-old Rad51cko/neo males, which were potentially infertile as identified by testes weight, and a Rad51cneo/+ littermate control. We found that although a few metaphase I spermatocytes (16%; n = 114) from mutant males appeared normal, similar to the control spermatocytes (Fig. 4, C and D), the majority (84%) were morphologically abnormal, possibly undergoing apoptotic fragmentation of the chromatin (Fig. 4, E and F). Unpaired autosomal univalents were visible in each of these spreads (Fig. 4, E and F; arrowheads). Such univalents are known to induce apoptosis at metaphase I (Eaker et al., 2001). Although metaphase I and II spermatocytes were almost equally represented in control animals (57% and 43%, respectively; n = 90), metaphase II spermatocytes were rarely found in mutant males (9%; n = 125). This suggests that mutant spermatocytes rarely progress beyond metaphase I, as they undergo apoptosis at this stage.

Meiotic defects in females

Ovaries from infertile females were similar in size compared with fertile littermates, and histological examination showed the presence of morphologically normal follicles at all stages of development (Fig. 5, A–F). However, ovaries from 6- and 12-wk-old animals revealed the absence of corpora lutea in infertile Rac51cko/neo females, suggesting that failure to ovulate may be the cause of infertility (Fig. 5, D and E). This ovulation block could be overcome by hormonal (intraperitoneal injection of 5 IU [0.1 cc] of pregnant mare serum [PMS] followed by a second injection of 5 IU [0.1 cc] of human chorionic gonadotropin [hCG] 48 h later) treatment (see ovary histology after superovulation; Fig. 5 F), after which infertile Rad51cko/neo females could become pregnant when mated with wild-type males. When embryos from the uterine horns of these mice were dissected at embryonic day 8.5, we found that the number of embryos obtained from infertile Rad51cko/neo females was greatly reduced (5 ± 0; n = 2) compared with the number of embryos from heterozygous Rad51cneo/+ superovulated littermates (15.5 ± 4.94; n = 2; P = 0.05). In addition, 7/10 embryos (70%) from the mutant females displayed a wide range of developmental abnormalities compared with only two abnormal embryos out of 31 (6.5%) from superovulated control mice (Fig. 5 H). All of the embryos from the control females appeared to develop normally (Fig. 5 G). We hypothesized that the developmental defects observed in these embryos resulted from gross chromosomal defects in the oocytes caused by abnormal meiosis (Yuan et al., 2002).

To determine the cause of such abnormalities in oocytes, we examined the meiotic progression of germinal vesicle (GV)–intact oocytes by in vitro maturation. Chromosomes of the control Rad51cneo/+ oocytes were aligned at the metaphase plate after 8 h of maturation (Fig. 5 I). Mutant oocytes progressed to metaphase I, and some aligned normally along the metaphase plate (Fig. 5 K). However, 60–75% (n = 50) of them displayed

![Figure 4](image-url)

**Figure 4.** Univalents are frequently found in mutant spermatocytes that progress to metaphase I. [A and B] SCP3 (green) and γ-H2AX staining (red) indicate the presence of a few completely unsynapsed chromosomes in infertile Rad51cko/neo spermatocytes as early as pachytene. Arrowheads mark unsynapsed chromosomes or univalents. [C–F] Metaphase I spreads of spermatocytes from control Rad51cneo/+ (C) and mutant males (D–F). A few mutant spermatocytes display proper pairing of homologous chromosomes (D), but the majority shows the presence of univalents (arrowheads in E and F). The abnormal appearance of chromosomes in the spreads containing univalents may indicate their apoptotic DNA degradation.

### Table I. Stage distribution of spermatocytes from Rad51cko/neo males in prophase I

| Genotype  | Leptotene | Early zygotene | Late zygotene/pachytene | Diplotene/diakinesis |
|----------|------------|----------------|-------------------------|----------------------|
| ko/ko     | 8%         | 21%            | 60% (mostly P)          | 11%                  |
| ko/neo    | 22%        | 30%            | 40% (mostly LZ)         | 8%                   |

Mutant spermatocytes undergo early arrest during prophase I. 184 and 208 spermatocytes stained for SCP3/SCP1 were counted in control and mutant samples, respectively. LZ, late zygotene; P, pachytene.
poorly structured metaphase plates (Fig. 5 J) in contrast to only 30% (n = 33) of abnormal oocytes from control Rad51cko/ko littersmates. The reason for the relatively high number of abnormal oocytes observed in control samples is unclear. One possibility is that this might be an effect of haplinsufficiency, as these animals were heterozygous for the hypomorphic allele.

We examined the karyotypes of metaphase I oocytes, which revealed no apparent defects in pairing and cohesion in infertile Rad51cko/ko females (n = 8) compared with the oocytes from Rad51cko/ko females (n = 6; Fig. 6, A and B). Evaluation of the centromeric cohesion between sister chromatids was facilitated by using a centromere-specific probe (Fig. S5, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200608130/DC1). We then karyotyped the oocytes that were allowed to progress to metaphase II in vivo after hormonal treatment. At metaphase II, oocytes from fertile females show the presence of 20 pairs of chromatids, each consisting of two sister chromatids that are attached together at their centromeres (Fig. 6 C). In oocytes from infertile Rad51cko/ko mice, we found a variety of chromosomal abnormalities. A majority of the mutant oocytes (85%; n = 34) showed precocious separation of sister chromatids (PSSC), indicating a problem with chromatid cohesion (Fig. 6 D). The degree of the cohesion defect varied from cases in which all chromosomes were affected (approximately half of all cases) to those in which only a few chromosomes were affected (for more examples, see Fig. S5, D–G). Only one oocyte from the control littermate was found to have a PSSC phenotype (5%; n = 20). Mutant oocytes with a PSSC phenotype often had a few acentric chromatids (40%; n = 10; Fig. 6 D, open arrowhead; and Fig. S5 F, arrowheads). On the other hand, a few other chromatids in the same spreads had two centromeres (Fig. 6 D, double arrows; and Fig. S5, E–G; double arrows). No such phenotype was observed in control oocytes. In addition, 20% of mutant oocytes (n = 10) in which chromosomes could be counted revealed >20 chromosomes or chromatin pairs, whereas others had 20 or fewer pairs of centromeres, indicating the abnormal segregation of homologous chromosomes during anaphase I of meiosis (Fig. S5 G).

The chromosomal defects observed in oocytes after metaphase I suggest a late role for RAD51C in recombinational repair. We did not find any evidence to support a direct role of RAD51C in sister chromatid cohesion (see Discussion; unpublished data). However, based on a biochemical study (Liu et al., 2004), the late function of RAD51C is likely to be associated with the resolution of HJs. To test whether HJ resolvase activity is indeed affected in Rad51c-deficient mouse cells, we examined protein extracts from mouse embryonic fibroblasts (MEFs) in an in vitro HJ resolution assay. Two Rad51cko/ko MEF lines were established on a p53-null background, which partially rescued the early embryonic lethality of Rad51c-null embryos (unpublished data). We used MEFs derived from p53-null and wild-type embryos as controls. HJ resolution activity of protein extracts from the two Rad51c mutant MEFs was at the background levels between 1.9 and 3.8% and did not correlate with the increasing amounts of protein extract (Fig. 6, E and F). The HJ resolution activity of the p53-null control cells increased from 5.4 to 11.3% as the amount of protein extract increased from 0.9 to 3.5 μg. This was slightly higher than the activity of the wild-type primary MEFs (between 1.0 and 7.5%; Fig. 6, E and F). These activity differences correlated with the amount of RAD51C protein detected in these extracts by Western blotting (Fig. 6 G). Thus, these data further support a RAD51C role in the resolution of HJs.

**Spermatocytes at metaphase II**

To test whether the premature separation of chromatids occurred even in males, we examined the spermatocytes that progressed to metaphase II (n = 39 in control and n = 11 in mutant samples). In control spermatocyte samples, we found 20 pairs of chromatids attached at the centromeres as observed in oocytes (Fig. 7 A and Fig. 6 C, respectively). Although the chromosomes in some mutant spermatocytes appeared to be normal (Fig. 7 B), others revealed fragmented chromosomes with broken centromeres or had aberrant chromosome numbers (Fig. 7, C and D). However, none of the mutant spermatocytes displayed the sister chromatid cohesion defect. Thus, the PSSC defect is a sexually dimorphic feature restricted only to females. It is unclear...
whether this phenotype in males is caused by a defect in DSB repair or is caused by the defect in HJ resolution. We present a model to explain how a defect in HJ resolution may result in PSSC in oocytes (see Discussion and Fig. 8).

**Discussion**

**Early role of RAD51C in mouse meiosis**

To date, the only well established function of RAD51C is its role in the process of homologous recombination by facilitating RAD51 foci formation after DNA damage. The precise role of RAD51C in the recruitment of RAD51 and how it differs from other RAD51 paralogues is still unclear. More importantly, recent studies of AtRad51c- and AtXrcc3-deficient Arabidopsis plants and fruit flies deficient in a RAD51C-like gene, spn-D, point to their unique requirement in meiosis unlike other RAD51 paralogues (Abdu et al., 2003; Bleuyard et al., 2005). Characterization of the AtRad51c mutant plants showed that this defect is associated with the repair of Spo11-induced DSBs, leading to abnormal synapsis and severe chromosomal fragmentation at the pachytene stage of prophase I (Abdu et al., 2003; Li et al., 2005).

In this study, we describe a meiotic defect in Rad51c mutant male mice that is associated with abnormal synapsis between homologous chromosomes at pachytene. At this stage, abnormal synapsis is indicated by the presence of γ-H2AX foci on some of the autosomal chromosomes. Occasionally, entire chromosomes appeared positive for γ-H2AX, indicating completely unsynapsed chromosomes (Fig. 4, A and B). Unlike Arabidopsis, mouse spermatocytes with unsynapsed chromosomes undergo apoptosis either at pachytene or metaphase I.
(Mahadevaiah et al., 2000; Eaker et al., 2001). Consistent with this, we observed a massive increase in abnormal metaphase I spermatocyte spreads.

Chromosome synapsis is dependent on RAD51-mediated recombination machinery, which helps bring homologous chromosomes together to repair DNA breaks introduced at leptotene using homologous regions as a template. We found that in Rad51cko/neo spermatocytes, the number of RAD51 foci was reduced about threefold as early as at leptotene. This is consistent with observations of attenuated RAD51 foci formation in RAD51C-deficient cell lines and with a recent finding that RAD51C may interact with RAD51 directly or as a complex with XRCC3 (Eaker et al., 2001; Rodrigue et al., 2006). A similar role for Rad55/Rad57, the budding yeast paralogues of RAD51, has been described previously (Gasior et al., 2001). The lack of RAD51 foci revealed in spermatocytes of Brca2-deficient mice results in developmental arrest at late zygote (Sharan et al., 2004). However, Rad51cko/neo spermatocytes do not completely arrest at this stage but clearly accumulate at leptotene and at early to midzygote. This suggests that the RAD51C defect in these mice causes impairment of the RAD51 function; however, because of the hypomorphic nature of the mutation, some spermatocytes escape an early arrest at zygote, progress further with unrepaired DSBs, and exhibit partially or completely unsynapsed chromosomes so that most of them are eventually blocked at metaphase I.

**Late role of RAD51C in mouse meiosis**

Like the males, a subset of the Rad51cko/neo females failed to produce any litters. Female infertility was associated with an ovulation failure, as no corpora lutea were found in ovaries of such mice. In ovaries of adult mice, oocytes are normally arrested in meiotic prophase I (Borum, 1961). As ovaries of the infertile Rad51cko/neo mice were of the same size as the wild-type animals and contained follicles at all stages of maturation, we conclude that Rad51c-deficient oocytes are able to progress to pachytene normally without an early arrest like in males.

However, oocytes from infertile Rad51cko/neo mice do suffer a maturation defect preventing ovulation, which could be overcome only by external hormonal stimulation. Although the number of GV-intact oocytes isolated from ovaries of such females was not considerably reduced compared with littermate controls, they often appeared more fragile to handle during the in vitro maturation experiments (unpublished data). Mutant oocytes display other early meiotic defects like an increased incidence of dysregulated chromosome alignment at the metaphase plate during metaphase I. Such defects may result from the impaired DSB repair as observed in mutant spermatocytes, but, unlike spermatocytes, oocytes could progress to metaphase I even with unrepaired DSBs as a result of sexually dimorphic checkpoint mechanisms (Eaker et al., 2001; Hunt and Hassold, 2002). Sexually dimorphic phenotypes in mice have been reported for several other meiotic mutations. Mutations in genes like Scp3, Mei1, and Brca2 result in male meiosis arrested during a zygote to pachytene transition, whereas mutant oocytes can progress through pachytene all the way to metaphase I (Libby et al., 2002; Yuan et al., 2002; Sharan et al., 2004). Similarly, sexual dimorphism in Rad51c-deficient mice is reflected by the fact that 37% of Rad51cko/neo males were infertile, whereas only 12% of females were infertile.

Despite a slightly dysregulated chromosome alignment at the metaphase plate, mutant oocytes were karyotypically...
normal and did not display unsynapsed chromosomes, which is characteristic of metaphase I oocytes with early meiotic dysfunctions (such as MLH1 and Mei1-deficient mice; Woods et al., 1999; Libby et al., 2002). Thus, abnormal embryos produced by infertile females after superovulation must be caused by defects occurring after the metaphase I stage. Indeed, major chromosomal abnormalities were found in mutant oocytes later at metaphase II such as PSSC, aneuploidy, and broken chromosomes.

Role in sister chromatid cohesion versus HJ resolution
PSSC is the most prominent defect at metaphase II, affecting almost all oocytes of the Rad51c mutant infertile females. The PSSC phenotype has been previously demonstrated for several genes regulating meiotic cohesion such as SMC1β in mice and Sgo1, Bub1, and PP2A in yeast (Bernard et al., 2001; Kitajima et al., 2004; Revenkova et al., 2004; Riedel et al., 2006). Sister chromatid cohesion is mediated by the multiprotein complex cohesin, comprising REC8, STAG3, SMC1β, and SMC3 proteins (Prieto et al., 2004). During prophase I of meiosis, cohesin is required for synaptonemal complex formation and recombination to occur between homologous chromosomes rather than between sister chromatids (Schwacha and Kleckner, 1997). Although cohesin is cleaved and released from chromosome arms by separase at metaphase I, Shugoshin protects it at centromeres until anaphase II (Fig. 8; Wang and Dai, 2005). This ensures a correct segregation of homologues at anaphase I and sister chromatids at anaphase II into separate daughter cells. Mice deficient for meiotic cohesins are infertile and display a wide range of defects, from the failure of interhomologous synopsis and recombination for Rec8−/− mice (Xu et al., 2005) to the occasional break-up of bivalents at metaphase I and premature sister chromatid separation that is detectable at metaphase I and is fully exposed at metaphase II for SMC1β−/− mice (Revenkova et al., 2004). This sister chromatid cohesion defect at metaphase II seen in infertile Rad51cko/neo−− females is remarkably similar to defects found in SMC1β knockout mice (Revenkova et al., 2004). Interestingly, a hamster cell line lacking functional RAD51C has also been reported to have defects in sister chromatid cohesion (Godthelp et al., 2002). Altogether, these findings suggested a possible role for RAD51C in sister chromatid cohesion.

We tested whether RAD51C protein can directly interact with proteins involved in sister chromatid cohesion like RAD21, REC8, SMC1β, and Shugoshin (Sgo1) by coimmunoprecipitation but found no evidence to support this hypothesis (unpublished data). This does not eliminate the possibility that RAD51C affects cohesins indirectly via other binding partners or signaling pathways. However, other evidence suggests that RAD51C may not be directly involved in sister chromatid cohesion. First, immunostaining of spermatocyte spreads for SMC1β, RAD21, and REC8 did not reveal any obvious abnormalities during prophase I in male meiosis (Fig. 3, N and P; and not depicted). Second, neither PSSC nor univalents were observed in Rad51c mutant oocytes at metaphase I unlike SMC1β−/− mice (Revenkova et al., 2004). Third, cohesin failure alone does not explain the presence of acenomeric chromatids and chromatids with two centromeres seen in the Rad51c−/− deficient oocytes. Fourth, the few mutant spermatocytes that reach metaphase II do not show any defect in sister chromatid cohesion (Fig. 7). Finally, no sister chromatid cohesion defects were found in Rad51c−/− embryos generated from Rad51c−/− embryos (unpublished data). On the other hand, in addition to biochemical activity, a role for RAD51C in HJ resolution is supported by its colocalization with MLH1 to the site of an obligate crossover at the pseudo-autosomal region of the sex chromosomes and by the observation that the number of RAD51C foci is substantially reduced in spermatocyte spreads of Mlh1−/− mice (Liu et al., 2007). Also, we have shown that protein extracts from Rad51c−/− MEFs lack HJ resolution activity, which further supports this finding (Fig. 6, E and F).

How does the HJ resolution defect result in PSSC?
If RAD51C indeed plays a role in the resolution of HJ, why do RAD51C mutant oocytes exhibit a sister chromatid cohesion defect? A precise phenotype of an HJ resolvase deficiency in the mouse is difficult to predict because so far no other mouse protein has been implicated in this process. Mus81-Eme1 was identified as an HJ resolvase in fission yeast (Boddy et al., 2001). Yeast cells deficient for any of these genes are sterile as a result of a defect in chromosome segregation. However, in higher eukaryotes, HJ resolution is likely to be performed by other molecules because Mus81-deficient mice are fertile and do not show any meiotic defect (Dendouga et al., 2005). We propose that the PSSC phenotype reflects the response of unresolved chromosomes to the increased tension exerted by the spindle at the kinetochores when the chromosomes are pulled to the opposite poles. This model is supported by the behavior of dicentric chromatids in mouse oocytes, which revealed a range of meiotic defects that were surprisingly similar to those of Rad51c−/− deficient oocytes, including PSSC as well as broken or acenomeric chromatids and aneuploidy at metaphase II (Koehler et al., 2002). Such dicentric chromosomes were generated in strains of mice that were heterozygous for an inversion in a large region of chromosomes X or 19. Meiotic recombination in the inverted region led to the generation of dicentric chromatids. When pulled in opposite directions during anaphase I, such chromatids resulted in PSSC in 90–97% of cases and rarely in broken chromosomes (3–10%). Interestingly, the meiotic behavior of such chromosomes in female mice was different from that in male mice, flies, and maize, which were more prone to breakage or selective loss (Koehler et al., 2002). Consistent with this observation, metaphase II spermatocytes from Rad51cko/neo−− mutant mice do not show any sister chromatid cohesion defect but do show chromosomes with broken centromeres (Fig. 7, C and D).

We speculate that a similar situation would be created if homologous chromosomes failed to dissolve chiasmata. Chiasmata are the cytological manifestation of crossovers, which are believed to arise from double HJs (Szostak et al., 1983). Therefore, we conclude that the late meiotic defects found in oocytes of infertile Rad51cko/neo−− mice can be associated with the HJ resolution failure and propose a model to explain its mechanism (Fig. 8).
Rad51cko/neo daughter cell, which would lead to aneuploidy detectable at that both homologous chromosomes may segregate into one to have originated from a sister chromatid. It is also possible affected. As shown in Fig. 6 D (inset), in several oocytes, we did that in such cases, the centromeric cohesion would remain un- broken chromosomes (Fig. 6 and Fig. S5, E–G). We predicted chromosome breakage is another consequence of increased arrest (Fig. 8).

We propose that in Rad51c-deficient oocytes, meiosis proceeds normally until anaphase I. However, there is an accumulation of recombination intermediates such as double HJs that hold the homologous chromosomes together even though the chiasmata fail to fully mature. At the onset of anaphase, there is an increase in tension at the centromere as a result of the persistence of unresolved double HJs. The increased tension may disrupt sister chromatid cohesion at the centromere, as has been demonstrated for dicentric chromosomes and the unpaired X chromosome of XO mice (Hodges et al., 2001; Koehler et al., 2002). The exact mechanism of this process is unclear. However, centromeric sister chromatid cohesion is sensitive to chemicals interfering with the metaphase I to anaphase I transition (Yin et al., 1998; Mailhes et al., 1999). In addition, Sgo1 has been shown to be a sensor of kinetochore tension in mitotic cells (Indjejikian et al., 2005). Although it remains to be shown in meiosis, it is likely that Shugoshin degradation/disruption caused by increased tension plays a critical role in the PSSC phenotype that is so prominent in Rad51c-deficient oocytes.

In addition to the disruption of centromeric cohesion, chromosome breakage is another consequence of increased tension. Indeed, half of all Rad51c-deficient oocytes displayed broken chromosomes (Fig. 6 and Fig. S5, E–G). We predicted that in such cases, the centromeric cohesion would remain unaffected. As shown in Fig. 6 D (inset), in several oocytes, we did observe single chromatids with an extra centromere that is likely to have originated from a sister chromatid. It is also possible that both homologous chromosomes may segregate into one daughter cell, which would lead to aneuploidy detectable at metaphase II. We did see aneuploidy in 2/10 oocytes in which individual chromosomes could be counted (Fig. S5 G).

Based on the meiotic defects observed in infertile Rad51clox/lox females, we speculate that RAD51c functions in late stages of meiotic recombination, possibly participating in the resolution of HJs. We cannot rule out the possibility that RAD51C may play a role in sister chromatid cohesion, which may explain the PSSC defect in oocytes. Similarly, it is possible that the primary defect may be the same (i.e., a defect in RAD51-mediated DSB repair) in males and females, but the phenotypes are different because of sexually dimorphic checkpoints. However, based on the evidence presented here, we have proposed a model connecting the impairment of HJ resolution function in Rad51clox/lox infertile females to the observed phenotype in oocytes.

### Materials and methods

**Generation of Rad51c mutant embryonic stem cells and mice**

The Rad51clox allele was generated in embryonic stem cells in which a neo resistance gene flanked by two loxP sites was inserted into intron 1 and a single loxP was inserted into intron 3. Heterozygous offspring in the C57BL/6 × 129/Sv mixed genetic background were crossed with β-actin–cre transgenic mice (Lewandoski et al., 1997) to obtain the Rad51clox/clox mice. Mutant Rad51clox/clox mice were generated by crossing Rad51clox/− or fertile Rad51clox/+ mice to Rad51clox/− mice.

**Fertility testing**

To determine the fertility status of Rad51clox/clox mice, 6-week-old males and females were mated with fertile animals for 4–10 wk. To monitor the mating behavior, mice were checked for vaginal plugs daily in a course of at least 6 wk. Mice that plugged several times but failed to produce any offspring were identified as infertile. Infertile Rad51clox/clox mice are referred to as mutant mice in the text for simplicity.

**Western blotting**

Protein lysates from control and mutant testes were prepared in cold radioimmunoprecipitation assay buffer. Samples containing 80 µg of protein were separated in NuPAGE 4–12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred onto a nylon membrane. The membrane was probed with mouse or rabbit α-RAD51C antibody (Novus Biologicals or Chemicon International, respectively) at 1:500 dilution or were probed with β-actin–cre antibody [NeoMarkers] diluted 1:400 according to standard procedures. Secondary α-mouse IgG-HRP antibody (1:2,000; Santa Cruz Biotechnology, Inc.) and an ECL chemiluminescence system (GE Healthcare) were used for signal visualization.

**Histology**

Testes and ovaries were fixed in Bouin’s solution. Samples were dehydrated through an ethanol series, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin. Slides were examined using brightfield microscopy. For TUNEL staining, testes were fixed in 10% neutral buffered formalin and were stained using the ApopTag kit (Chemicon International) according to the manufacturer’s instructions.

**Spermatocyte spread preparation and immunofluorescence**

Surface spreads of spermatocytes from the testes of mutant and control animals were prepared and stained as described previously (Romanienko and Camerini-Otero, 2000). Another method was used to prepare spermatocytes for the RAD51 and RPA staining and was described previously (Counce and Meyer, 1973). The difference between the two protocols is that no enzymatic treatment is involved in the second method, and cells are separated by pipetting and spread in a hypotonic solution directly on a slide. The following primary antibodies were used for immunofluorescence: rabbit anti–γH2AX (1:1,000; obtained from W. Bonner, National Cancer Institute, Bethesda, MD), mouse anti–MLH1 (1:10; BD Biosciences), rabbit polyclonal α-SCEP (1:500), α-SCEP1 (1:1,000), α-RPA (1:100; all provided by R. Moens, York University, Toronto, Canada), mouse monoclonal α-SMC1β (1:10; provided by E. Revenkova, Mount Sinai School of Medicine, New York, NY), and rabbit α-Rad51 (1:500; obtained from S. West, Cancer Research UK, South Mimms, UK). Secondary antibodies used were goat anti-rabbit AlexaFluor488, goat anti-rabbit AlexaFluor568, goat anti–mouse AlexaFluor488, and goat anti–mouse AlexaFluor568 (Invitrogen). Secondary antibodies were used at a 1:250 dilution.

**Image acquisition**

Images were acquired with a microscope (Axioplan 2; Carl Zeiss Micro Imaging, Inc.) using an oil plan Neofluar 100× 1.3 NA objective (Carl Zeiss Microimaging, Inc.). Images were taken with a CCD camera (Quantix; Photometrix) and processed using SmartCapture software (Desksoft). Images were further processed with Photoshop software (Adobe) to adjust for size and contrast.
Superovulation and collection of oocytes and embryos
Females were superovulated, and oocytes and embryos were collected as described previously (Hogan et al., 1994). In brief, 0.1 ml PMS (5 IU; Sigma-Aldrich) was injected intraperitoneally into female mice. GV-intact stage oocytes were collected 44–48 h later in flushing and holding medium with 3 mg/ml BSA (Specialty Media) by puncturing ovaries with a 27-gauge needle. Oocytes were incubated briefly in 0.1 mg/ml hyaluronidase type IV S (Sigma-Aldrich), and cumulus cells were removed by pipetting before transfer into Chatot, Ziemek, and Bavister media containing 50 μg/ml BSA (Specialty Media) for 8 h at 37°C and 5% CO2 to obtain metaphase I stage oocytes for immunocytochemistry and karyotyping. Alternatively, 48 h after PMS treatment, 6-wk-old female mice were given an intraperitoneal injection of 0.1 ml hCG (5 IU; Sigma-Aldrich). 14 h later, metaphase II-stage oocytes were collected from ampullae. To obtain embryos, 6-wk-old female mice given both PMS and hCG injections were placed with stud males.

Immunocytochemistry of oocytes
Metaphase I and II stage oocytes were washed in Dulbecco’s PBS without CaCl2 or MgCl2 (Invitrogen) and were fixed for 10 min in cold methanol. Oocytes were incubated in blocking buffer (4% BSA; Sigma-Aldrich), 10% normal goat serum (Vector Laboratories) overnight at 4°C, monoclonal anti-β-tubulin clone TUB2.1 (Sigma-Aldrich) for 1 h at RT, AlexaFluor488 goat anti–mouse IgG (Invitrogen) for 1 h at RT, and in a 1:5,000 dilution of 1 mg/ml DAPI (Roche) for 20 min at RT before mounting in Vectashield Mounting Medium (Vector Laboratories) on Shandon Multi-Spot microscope slides (Thermo Savant).

Karyotyping
Metaphase spreads of spermatocytes were prepared by the Evans method from the testes of 3-wk-old mutant and control males (Evans et al., 1964). Oocytes were karyotyped by an air-drying method as described previously (Tarkowski, 1966). In brief, mutant and control females were injected with PMS followed by hCG as described above (see Superovulation and collection of oocytes and embryos), and, 14 h later, oocytes arrested at metaphase II were collected from the ampullae in Weimouth media containing penicillin-streptomycin, 10% FBS, and 2.5 mg/ml sodium pyruvate. Chromosome spreads were prepared from the oocytes after a 2-min incubation in 0.9% sodium citrate solution. H1 resolution assay
Protein extraction was performed as follows: exponentially growing MEFS were collected from ~20 15-cm tissue culture dishes after trypsinization. Approximately 1 g of a cell pellet was resuspended in prechilled lysis buffer (10 mM Tris, pH 8.0, 1 M KCl, 1 mM EDTA, and 1 mM DTT) in the presence of complete EDTA-free protease inhibitor cocktail (Roche). Cells were broken open by low speed centrifugation (30 min at 9,000 rpm). The (NH4)2SO4 was added to the supernatant and the insoluble pellet was removed by centrifugation at 13,000 rpm for 1 h. Approximately 1 g of protein was assayed by the Lowry method.

Online supplemental material
Fig. S1 shows aberrant splicing of the Rad51c transcript as a result of the presence of the PGK-neo cassette. Fig. S2 shows quantitative analysis of MLH1 foci formation in spermatocytes from control and infertile Rad51cko/neo mice. Figs. S3 and S4 show quantitative analysis of RPA foci (Fig. S3) and RAD51 foci (Fig. S4) in spermatocytes from control and infertile Rad51cko/neo mice. Fig. S5 shows chromosomes from control and Rad51cko/neo oocytes at metaphase I hybridized with a pancentromeric probe as well as sister chromatid cohesion and other defects at metaphase II. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200608130/DC1.

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