DEVELOPMENT AND DISEASE

Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice

Xiaoling Xu1,2,*, Olga Aprelikova3, Peter Moens4, Chu-Xia Deng2 and Priscilla A. Furth1,5

1Department of Physiology, University of Maryland School of Medicine, Baltimore 21201, USA
2Genetics of Development and Disease Branch, 10/9N105, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA
3Laboratory of Biosystems and Cancer, 37/5016, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
4Department of Biology, York University, Toronto, Ontario, M3J 1P3, Canada
5Department of Oncology, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007, USA

*Author for correspondence (e-mail: xiaolingx@intra.niddk.nih.gov)

Accepted 24 January 2003

SUMMARY

Breast tumor suppressor gene 1 (BRCA1) plays an essential role in maintaining genomic integrity. Here we show that mouse Brca1 is required for DNA-damage repair and crossing-over during spermatogenesis. Male Brca1<sup>-/-<sup>Δ11p53+/-</sup></sup> mice that carried a homozygous deletion of Brca1 exon 11 and a p53 heterozygous mutation had significantly reduced testicular size and no spermatozoa in their seminiferous tubules. During spermatogenesis, homologous chromosomes from the mutant mice synapsed and advanced to the pachytene stage but failed to progress to the diplotene stage. Our analyses revealed that the Brca1 mutation affected cellular localization of several DNA damage-repair proteins. This included prolonged association of γH2AX with sites of DNA damage, reduced sex body formation, diminished Rad51 foci and absence of Mlh1 foci in the pachytene stage. Consequently, chromosomes from mutant mice did not form chiasmata, a point that connects exchanging homologous chromosomes. Brca1-mutant spermatocytes also exhibited decreased RNA expression levels of several genes that are involved in DNA-damage repair, including RuvB-like DNA helicase, XPB, p62 and TFIID. Of note, the premature termination of spermatogenesis at the pachytene stage was accompanied by increased apoptosis by both p53-dependent and p53-independent mechanisms. Thus, our study revealed an essential role of Brca1 in DNA-damage repair and crossing-over of homologous chromosomes during spermatogenesis.

Key words: BRCA1, MLH1, γH2AX, Crossing-over, Apoptosis

INTRODUCTION

Mammalian spermatogenesis proceeds through well-defined phases of mitotic and meiotic divisions as spermatogonia develop into spermatocytes, round spermatids and, finally, spermatozoa. In mice, all cell division in the testis is mitotic division before postnatal day 7 (P7). Starting from P8, spermatogonia enter the meiotic prophase of the meiotic pathway and homologous chromosomes undergo a programmed sequence of compaction, synopsis, recombination and segregation that leads to a reduction of genetic material from diploid to haploid (reviewed by Cohen and Pollard, 2001; Tarsounas and Moens, 2001). A unique feature of meiosis I prophase chromatin is a high frequency of DNA double-strand breaks (DSBs), which are required for recombination between homologous chromosomes (reviewed by Cohen and Pollard, 2001; Roeder, 1997). Repair of DSBs is necessary to maintain genome integrity and limit the mutational potential of these breaks. Loss-of-function mutations of many factors involved in DNA damage repair result in defective spermatogenesis (reviewed by Cohen and Pollard, 2001; Dasika et al., 1999; Tarsounas and Moens, 2001).

Germline mutations of BRCA1 are found in approximately half of familial breast cancer cases and the majority of kindreds with combined familial breast and ovary cancer (Alberg and Helzlsouer, 1997; Brody and Biesecker, 1998; Paterson, 1998). BRCA1 contains 24 exons that encode proteins of 1863 and 1812 amino acids in human and mouse, respectively (Lane et al., 1995; Miki et al., 1994). It has been shown that BRCA1 contains multiple functional domains that interact with numerous molecules including products of tumor suppressor genes, oncogenes, DNA damage-repair proteins, cell-cycle regulators, ubiquitin hydrolases, and transcriptional activators and repressors (reviewed by Deng and Brodie, 2000). These observations indicate that BRCA1 plays important roles in...
Brca1 gestation, they can survive to adulthood if one or both Brca1 alleles was utilized to test this hypothesis. Although Brca1 mutation of survive to adulthood in a DNA-damage repair.

Significantly, mice homozygous for a hypomorphic mutation of Brca1 survive to adulthood in a p53–/– mouse Brca1 reveal essential functions of this gene in multiple DNA damage-repair pathways. This includes nuclear excision repair (Hartman and Ford, 2002), transcription-coupled repair of oxidative DNA damage (Abbott et al., 1999; Gowen et al., 1998), homologous recombination repair (Moyzahan et al., 1999; Moyzahan et al., 2001; Snouwaert et al., 1999) and nonhomologous end-joining (Baldeyron et al., 2002; Zhong et al., 2001; Snouwaert et al., 1999). Studies of somatic cell lines carrying mutations in either human BRCA1 or mouse Brca1 reveal essential functions of this gene in multiple DNA damage-repair pathways. This includes nuclear excision repair (Hartman and Ford, 2002), transcription-coupled repair of oxidative DNA damage. (Abbott et al., 1999; Gowen et al., 1998), homologous recombination repair (Moyzahan et al., 1999; Moyzahan et al., 2001; Snouwaert et al., 1999) and nonhomologous end-joining (Baldeyron et al., 2002; Zhong et al., 2002a; Zhong et al., 2002b). In conclusion, genetic instability associated with BRCA1 deficiency is caused, at least in part, by impaired DNA-damage repair.

Materials and methods

Testis preparation

Brca1+/–/+, Brca1+/–/+ and Brca1–/– mice were maintained in a mixed genetic background of 129/FVB/Black Swiss (Xu et al., 2001). BrdU was injected 2 hours prior to euthanasia. Testes were fixed in either Bouin’s (for immunofluorescence studies) or 10% formalin (for BrdU labeling) for either 4 hours or overnight. Tissues were dehydrated in increasing ethanol concentrations (70%, 95%, 100%) for 30 minutes each. Then they were treated 2 times with xylene (10 minutes each time), 1 time with a xylene/wax mixture at 50°C (1 hour), 3 times with wax alone (1 hour each time), followed by embedding in paraffin and sectioning at a thickness of 4-6 μm. Sections either were stained with Hematoxylin and Eosin (H&E) or processed for immunohistochemical analysis using antibodies to germ cell nuclear antigen (GCNA) (1:50, a kind gift from Dr George Enders, University of Kansas Medical Center) or to antiphospho-histone H3 (Upstate, Biotechnology, Lake Placid, NY). Slides were examined and photographed under a light microscope.

Preparation of chromosome spreads for immunofluorescence

Unrepaired DNA damage.

Western blotting

Western blots were carried out using standard procedures using the following antibodies: goat anti-DMC1 at 1:1000 dilution (Santa Cruz Biotechnology); rabbit anti-RAD51 at 1:1000 dilution (Santa Cruz Biotechnology); mouse anti-MLH1 at 1:500 dilution; and mouse anti-Pms2 at 1:1000 dilution (Pharmingen, San Diego, CA).

Semiquantitative RT-PCR

Total RNA was extracted from testes at different developmental stages, including P14, P16, P21 and P22. Reverse-transcription reactions were carried out using a 1st strand cDNA-synthesis kit (Roche, Indianapolis, IN). The cDNA samples were stored at −20°C. One μg of RNA from each sample was used as template for each reaction and 1 μl of cDNA from each sample was used for PCR reaction. The optimal number of cycles for amplification was determined according to the cycle number that yielded the strongest band in the linear range. The ranges of cycles varied from 25-28, 30-35, 35-40 for different primer sets.
depending on the specific RNA target and primer set. The samples were heated to 94°C for 2 minutes and then run through 25-28 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 minute, followed by 72°C for 10 minutes and then 4°C. Primers used in this study and the DNA lengths amplified are shown below.

TFIIH amplification (680 bp):
5’-cagaaaatcagtccagaggg-3’, 5’-ccctcgtctaatggcttatc-3’

TFIID amplification (360bp):
5’-gttgaaacagacatggtttgc-3’, 5’-cagccatgctaagaaagctac-3’

RuvB amplification (540 bp):
5’-gacacaccattcacagccatc-3’, 5’-gatctgttctcggacttctg-3’

GAPDH amplification (300 bp):
5’-acagccgcatcttcttgtgc-3’, 5’-tttgatgttagaggggtctgc-3’

TUNEL assay and BrdU-incorporation assay
Testis sections were analyzed for apoptosis using the ApoTag kit as recommended by the manufacturer (Interjen Company, Purchase, NY). To evaluate cellular proliferation rates, BrdU incorporation was measured using a cellular proliferation kit following the manufacturer’s directions (Amersham Bioscience, England).

γ-Irradiation
Mice at 1 month of age were irradiated at 80 Gy (1Gy minutes⁻¹, Gammacell 40) and were euthanized 10 minutes, 30 minutes, 2 hours and 4 hours after irradiation. Testes were removed and processed for chromosome spreads and immunofluorescence staining as described above.

Statistical analyses
Student’s T test was used to compare differences of pachytene and diplotene spermatocytes, rates of apoptosis, numbers of foci for Mlh1 and Rad51, and numbers of sex bodies for H2AX between Brca1 mutant and control mice at ages specified in the text. P<0.05 was considered statistically significant.

RESULTS
Spermatogenesis is defective in Brca1Δ11/Δ11 p53+/− male mice
Mature (26 weeks of age) Brca1Δ11/Δ11 p53+/− male mice (n=10) contained no spermatozoa in their testes (Fig. 1B, and data not shown). To identify the earliest stages at which defective spermatogenesis occurred, testes from P10-28 Brca1Δ11/Δ11 p53+/− mutant and p53+/− control mice were isolated and compared. At P10, testes of both Brca1 mutant and control mice were similar in size (Fig. 1C). Histological analyses demonstrated no obvious differences in seminiferous tubule size and the numbers of spermatogonial cells (Fig. 1D,E). However, starting from P16, testes from Brca1Δ11/Δ11 p53+/− mutant mice were significantly smaller than controls (Fig. 1F,I,L). In control mice, spermatocytes began to enter the diplotene stage at P16 and spermatocyte size increased (arrow, Fig. 1G), but no diplotene spermatocytes were detected in the testes from Brca1 mutant mice (Fig. 1H). At P21, spermatocytes from control mice had finished meiosis and formed spermatids (arrow, Fig. 1J). No spermatids were found in the testes from Brca1 mutant mice (Fig. 1K), instead, cells with dark Hematoxylin-stained nuclei were found (arrow, Fig. 1K). This phenotype was apparent in the testes of Brca1 mutant males from P28 to P90 (Fig. 1B,N and data not shown). These results indicate that spermatogenesis, including the first wave of spermatogenesis, did not progress beyond meiosis I in the Brca1 mutant mice.

Spermatogenesis is terminated prior to the diplotene stage of meiosis I in Brca1 mutant males
Molecular markers were used to define the stages of meiosis I that were defective in the Brca1 mutant male mice. GCNA is expressed specifically in premeiotic cells up to the pachytene

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Size and histology of testes from p53+/− control (C) and Brca1Δ11/Δ11 p53+/− mutant (M) mice. Ages of mice are indicated. (A,D,G,J,M) Control mice; (B,E,H,K,N) Brca1 mutant mice. No significant difference in size was detected between control and mutant testes at postnatal day 10 (P10); however, mutant testes are smaller at P16 and older. Arrows indicate spermatozoa (A), diplotene spermatocytes (G), round spermatocytes (J) and differentiated spermatids (M). Mutant testes lack these cells. Arrowheads in H,K,N indicate condensed nuclei, which are TUNEL positive (see Fig. 3).
The presence of prominent dark Hematoxylin-stained spermatocyte nuclei in the testes of Brca1 mutant mice (Fig. 1K) suggested that the Brca1 mutation caused increased rates of spermatocyte apoptosis. Tissue sections from testes of Brca1<sup>11/11</sup>+/− and p53<sup>−/−</sup> control mice at P10, P16 and P21 were analyzed by TUNEL assay to quantify these differences. At P10, <2% of seminiferous tubules in control and mutant testes contained apoptotic cells. This did not change in testes from control mice at P16 and P21. However, in Brca1 mutant mice the number of seminiferous tubules that contained apoptotic spermatocytes increased significantly at P16 and P21 (22% and 30%, respectively) (Fig. 3A). To directly compare rates of cellular apoptosis, the percentage of apoptotic cells were determined in TUNEL-positive seminiferous tubules that were selected randomly from testes of ten control and ten Brca1 mutant mice. At P10, in testes from both control and Brca1 mutant mice fewer than 4% of cells were apoptotic (Fig. 3B). At P16 and P21 the percentage of apoptotic cells did not change in testes from control mice, but increased significantly to 40% at P16 and 50% at P21 (both 0.0001 compared to control mice) in testes from Brca1 mutant mice (Fig. 3B). Thus, the premature termination of spermatogenesis at the pachytene stage of meiosis I in Brca1 mutant mice was accompanied by significantly increased rates of apoptosis.

Because activation of p53 induces apoptosis in a number of tissue/organ systems in Brca1<sup>11/11</sup>+/− mutant mice (Xu et al., 1999b; Xu et al., 2001), we next determined whether the increased rate of apoptosis in the Brca1 mutant testes was p53 dependent. Because only a few (1-2%) of Brca1<sup>11/11</sup>+/− male mice survive to adulthood (Xu et al., 2001), rates of apoptosis were compared in testes isolated from Brca1<sup>11/11</sup>+/−, Brca1<sup>11/11</sup>+/− and Brca1<sup>11/11</sup>+/− mice. TUNEL-positive cells were detected in testes of all three genotypes, indicating that the loss of p53 did not prevent apoptosis. In testes isolated from Brca1<sup>11/11</sup>+/− mice at P19 (Fig. 3C,D), the absolute numbers of apoptotic cells in each tubule was less than those found in testes from Brca1<sup>11/11</sup>+/− and Brca1<sup>11/11</sup>+/− mice. However, all
the tubules were nearly empty, suggesting either that more extensive cell death happened earlier in spermatogenesis or that germ cells did not robustly colonize the gonads during early development. A direct comparison between Brca1<sup>Δ/Δ</sup> p53<sup>–/+</sup> (Fig. 3E,F) and Brca1<sup>Δ/Δ</sup> p53<sup>–/–</sup> (Fig. 3G,H) testes demonstrated significant lower rates of apoptosis in p53<sup>–/–</sup> mice than in p53<sup>–/+</sup> mice. In ten, randomly selected TUNEL-positive seminiferous tubules from Brca1<sup>Δ/Δ</sup> p53<sup>–/+</sup> (P19) (C), Brca1<sup>Δ/Δ</sup> p53<sup>–/–</sup> (P21) (E) and Brca1<sup>Δ/Δ</sup> p53<sup>–/–</sup> (P21) (G) testes. Corresponding H&E-stained histological images are shown in D,F,H.

The results indicated that Brca1 mutation triggered apoptosis through p53-dependent and p53-independent mechanisms.
The majority of homologous chromosomes from the testes of Brca1 mutant mice are able to synapse but do not form crossing-overs

To determine whether or not the homologous synopsis process was impaired by Brca1 mutation, testicular chromosome spreads were stained with an antibody to synaptonemal complex protein 3 (SCP3). SCP3 is one of the components of the axial and lateral element of the synaptonemal complex. It appears in leptotene stage spermatocytes and disappears in late meiotic cells (Dobson et al., 1994). Analysis of >2000 spermatocytes from Brca1<sup>Δ11/Δ11</sup>/p53<sup>+/−</sup> and control mice at P10-P21 demonstrated that the chromosomes from spermatocytes of Brca1 mutant mice behaved normally until the pachytene stage (Fig. 4A-D). Only 3% of the cells showed delayed pairing of one or two chromosomes in Brca1 mutant mice (data not shown). Chromosomal spreads prepared from P16 Brca1 mutant and control mice demonstrated that approximately one third of spermatocytes of both strains completed synapses of homologous chromosomes (Fig. 4G). These observations were consistent with our earlier histological findings demonstrating that mutant spermatocytes developed relatively normally until the pachytene stage.

During normal spermatogenesis, diplotene stage spermatocytes begin to appear at P16 and represent 20-25% of the total spermatocyte population at P21 (Fig. 4E). A hallmark of chromosome behavior at these stages is the extensive separation of homologous chromosomes except for the crossing-over sites that are held by chiasmata (arrows, Fig. 4E). By contrast, few homologous chromosomes demonstrated separation in the spermatocytes from Brca1 mutant mice and those few spermatocytes that were not already eliminated through apoptosis showed fragmented cores/synaptonemal complexes (Fig. 4F). In fact, <1% of spermatocytes from Brca1 mutant mice exhibited even partial separation of their chromosomes (Fig. 4G). These observations demonstrated that spermatocytes from Brca1 mutant mice were arrested at the late pachytene stage and failed to enter the diplotene stage.

Absence of the Brca1 full-length isoform diminishes Mlh1 foci on chromosomes

Many DNA damage-repair proteins, including MLH1, RAD51 and DMC1, are involved in the crossing-over process. To investigate the molecular mechanisms underlying the failure of crossing-over formation in spermatocytes from Brca1 mutant mice, the expression patterns and sites of cellular localization of Mlh1, Rad51 and Dmc1 were determined.

In control mice, Mlh1 first appeared as distinct spots along the chromosomes of late zygote spermatocytes and was maintained until the mid-pachytene stage in spermatocytes (Fig. 5A, n=267). By contrast, no Mlh1 foci were observed on the majority (156 out of 162) of chromosome spreads prepared from late zygote to mid-pachytene stage spermatocytes of Brca1 mutant mice. The remaining six spermatocytes showed very weak staining (Fig. 5B). Western blotting was performed to determine whether the abnormal distribution of Mlh1 on the chromosomes from Brca1 mutant mice was due to reduced protein concentrations of either Mlh1 or its binding partner Pms2. There were no obvious differences in the concentrations of these proteins between Brca1 mutant and control mice at any of the stages examined (Fig. 5D). In conclusion, although the distribution of Mlh1 on chromosomes was abnormal in Brca1 mutant mice, overall expression levels were normal. Because Brca1 mutant females were fertile, localization of Mlh1 was also checked in female mice. Nearly all of the chromosomes isolated from 200 late zygote to mid-pachytene stage oocytes taken from E16.5-17.5 Brca1<sup>Δ11/Δ11</sup>/p53<sup>+/−</sup> female embryos had Mlh1 foci. There were no obvious differences in either the localization pattern or intensity of the foci between Brca1 mutant (Fig. 5C) and control (not shown) oocytes.
Abnormal localization of Rad51, but not Dmc1, in Brca1 mutant spermatocytes

Rad51 and Dmc1 are homologs of Escherichia coli RecA. They bind to DSBs and are involved in meiotic recombination repair (Masson and West, 2001). Because BRCA1 colocalizes with RAD51 on meiotic chromosomes and DMC1 colocalizes with RAD51 (Masson and West, 2001; Scully et al., 1997), we tested whether Brca1 mutation affected localization of these proteins. In control spermatocytes, Rad51 foci started to appear on the chromosomes of leptotene-stage spermatocytes (Fig. 6A, n=176) and disappeared on chromosomes from mid-pachytene-stage spermatocytes (data not shown). Analyses of 211 spermatocytes from three Brca1 mutant mice demonstrated significantly diminished numbers of Rad51 foci on all chromosomes (Fig. 6B). Western blots were performed to see if the failure to detect Rad51 on chromosomes was caused by reduced Rad51 protein concentrations. This analysis revealed comparable levels of Rad51 in Brca1 mutant and control animals (Fig. 6E), suggesting that the absence of full-length Brca1 isoform affected the localization of Rad51 but not the abundance. There were no apparent differences in Dmc1 localization and concentration between Brca1 mutant and control mice on chromosomes isolated from >300 spermatocytes (Fig. 6C-E).

The localization pattern of γH2AX is altered in spermatocytes from Brca1 mutant mice

H2AX is a member of the mammalian histone H2A family (reviewed by Redon et al., 2002). It is phosphorylated and relocated to DSBs within minutes of irradiation, which is consistent with a crucial role for this gene in DSB repair (Paull et al., 2000). H2AX-deficient mice display defective spermatogenesis and diminished numbers of Brca1 chromosomal foci (Celeste et al., 2002). To determine if Brca1 mutation alters H2AX phosphorylation and localization, chromosome spreads from P18, P21 and P28 testes of Brca1 mutant (n=6) and control (n=6) mice were prepared and subjected to double staining with an antibody to SCP3 and an antibody specific for phosphorylated H2AX (γH2AX). In control mice, we observed changes in γH2AX staining at different stages of spermatogenesis. It appeared first as diffused staining in leptotene-stage spermatocytes (Fig. 7A) but evolved into distinctly stained foci on chromosomes from late leptotene and early zygotene-stage spermatocytes (Fig. 7B). Beginning at the late zygotene stage, staining was restricted to the sex (XY) body and became gradually brighter and more condensed (arrow, Fig. 7C) until γH2AX localization was restricted to the sex body in almost all the cells examined (98%, 288 out of 304) throughout the pachytene stage (Fig. 7D). In Brca1 mutant mice, the pattern of γH2AX localization was similar to control mice at the leptotene and early zygotene stages (Fig. 7F,G). However, at the late zygotene and early pachytene stages, >90% (388 out of 427) of spermatocytes failed to show localization of γH2AX to the XY body. Instead, it remained as multiple distinct foci (Fig. 7H,I). The intensity of staining in the 10% of cells that demonstrated γH2AX localization in the XY body was decreased significantly (data not shown). The differences in the percentages of spermatocytes that showed γH2AX staining in the presence and absence of Brca1 mutation was highly significant (P<0.0001). This indicates that the localization pattern of γH2AX was disrupted by Brca1 mutation, similar to the disruptions of the Mlh1 and Rad51 patterns of localization.

Next we investigated possible reasons for the prolonged...
2008

presence of γH2AX foci on the chromosomes and the absence of γH2AX in the XY body. Because γH2AX detects and binds to DSBs (Mahadevaiah et al., 2001), we suspected that the prolonged presence of γH2AX foci in mutant cells could be caused by the accumulation of unrepaired DSBs. We further hypothesized that if sufficient DNA damage could be caused in control mice, they might exhibit defects similar to those observed in Brca1 mutant mice. To test this, we irradiated control and Brca1 mutant mice with 80 Gy of γ-irradiation to introduce acute, extensive DNA damage, and examined the formation of γH2AX foci at various time points post irradiation. Spermatocytes were isolated from the irradiated mice at 10 minutes, 30 minutes, 2 hours and 4 hours following irradiation. All irradiated p53+/− spermatocytes demonstrated prolonged γH2AX foci formation without XY body staining, similar to that found in the spermatocytes from unirradiated Brca1 mutant mice (Fig. 7D). There were no differences in the numbers of γH2AX foci in spermatocytes from irradiated Brca1 mutant mice compared to those from irradiated control mice (Fig. 7H).

Steady-state RNA levels of RuvB-like DNA helicase, XPB, p62 and TFIID are reduced in Brca1 mutant spermatocytes

Unwinding of parental DNA strands is a prerequisite for DNA replication, repair and recombination. The unwinding of duplex DNA is catalyzed by DNA helicases, which destabilize the hydrogen bounds between complementary double strands of DNA (Kanemaki et al., 1999). The absence of crossing-over in Brca1Δ1/Δ1p53+/− mutant spermatocytes prompted us to measure the expression of genes that encode proteins with DNA helicase, branch migration and basic transcription activity. RNA samples from P14, P16, P21 and P22 were prepared from p53+/− control and Brca1Δ1/Δ1p53+/− mutant mice. An appropriately sized RT-PCR product for p47, which encodes a RuvB-like DNA helicase, was detected in testes from both control and Brca1 mutant mice. In RNA isolated from control testicular tissue, the RNA encoding p47 began to increase at P16 and was maintained at high levels through P22 (Fig. 8A). By contrast, the concentration of p47 RNA did not increase in testicular tissue from Brca1 mutant animals (Fig. 7H).

Fig. 7. Absence of the Brca1 full-length isoform disrupted γH2AX localization. Scp3 and γH2AX double immunofluorescence localization of control and mutant spermatocytes. (A-D,F-I) Localization of γH2AX in unirradiated testes. Localization is similar at the leptotene (Lep) and early zygotene (E. Zyg) stages in control and mutant spermatocytes. Starting from the late zygotene (L. Zyg) stage, γH2AX localizes to the XY body in wild-type spermatocytes (arrows, C,D). However, in 90% of spermatocytes from Brca1 mutant mice, γH2AX fails to localize to the XY body and remains as multiple foci at the late zygotene stage (H) and early pachytene (E. Pac) stage (I). (E,J) Localization of γH2AX in early pachytene stage spermatocytes 10 minutes after 80 Gy irradiation. The XY body cannot be detected.
of full-length Brca1 and lower expression levels of p47, p62, XPB and TFIID, which is consistent with the notion that Brca1 is a positive regulator of these genes.

**DISCUSSION**

This study demonstrated an essential role for full-length Brca1 in spermatogenesis. Brca1 is required either directly or indirectly for repair of the double-stranded breaks that are introduced during the normal process of spermatocyte meiosis. By contrast, Brca1 does not appear to be required for oocyte meiosis. In Brca1 mutant mice spermatogenesis did not advance beyond the pachytene stage of prophase during meiosis I and was accompanied by increased rates of apoptosis. Prophase of meiosis I involves in recombination between homologous chromosomes (reviewed by Cohen and Pollard, 2001; Roeder, 1997). This process requires extensive DNA damage-repair activity because defective meiosis can result from the individual loss-of-function of several different factors involved in DNA-damage repair (reviewed by Cohen and Pollard, 2001; Dasika et al., 1999; Tarsounas and Moens, 2001).

The majority of homologous chromosomes synapsed normally at the pachytene stage in spermatocytes isolated from Brca1 mutant mice, indicating that Brca1 is not required for this process. However, spermatocytes from Brca1 mutant mice were arrested at the late pachytene stage and failed to enter the diplotene stage. This defect correlated with the absence of Mlh1 foci on the homologous chromosomes. Because Mlh1 foci localize to crossing-over sites on meiotic chromosomes and serve as a molecular marker for this event (Baker et al., 1996; Moens et al., 2002), it appears that full-length Brca1 is required for crossing-over. Crossing-over is a multi-step process that is initiated by a DSB in DNA, followed by exonucleolytic digestion, strand invasion, Holliday junction formation, branch migration and junction resolution, and, finally, heteroduplex DNA formation (reviewed by Cohen and Pollard, 2001; Roeder, 1997). It is possible that Brca1 is not required for the early steps of crossing-over because the phenotype of Brca1-deficient spermatocytes appeared at a later stage than those reported for Spo11-deficient (Romanenko and Camerini-Otero, 2000), ATM-deficient (Barlow et al., 1998), DMC1-deficient (Yoshida et al., 1998) and Msh4-deficient (Kneitz et al., 2000) spermatocytes.

Our data indicate a link between BRCA1 function and the proper localization of MLH1. Previous investigations revealed that MLH1, together with either MSH4/MSH5/PSM2 or MSH4/MSH5/MLH3, are involved in both mismatch repair and crossing-over (Cohen and Pollard, 2001). BRCA1 also displays an affinity for branched DNA structures and forms protein-DNA complexes cooperatively between multiple DNA strands without DNA sequence specificity (Paull et al., 2001). The results of the experiments presented here are consistent with the possibility that BRCA1 acts to recruit MLH1 to sites of recombination.

There are both similarities and differences between the phenotypes of Brca1 and Mlh1 deficient spermatocytes (Baker et al., 1996; Eaker et al., 2002). In both strains, the majority of homologous chromosomes undergo synopsis. A 10-100 fold reduction in chiasma formation is found in Mlh1 mutant mice.
whereas <1% of spermatocytes in Brca1 mutant mice show any sign of chiasma formation and spermatocytes deficient in either protein have high rates of apoptosis. However, although Brca1 mutant spermatocytes died at the end of the pachytene stage, the majority of Mlh1 mutant spermatocytes die during metaphase (Eaker et al., 2002). It is interesting to note that the effect of Brca1 mutation on formation of Mlh1 foci is male specific because Mlh1 foci are formed normally in oocytes.

In an attempt to further define the interaction between BRCA1 and MLH1, we performed co-immunoprecipitation experiments between BRCA1 and MLH1 in human spermatocytes but could not detect any direct binding between these proteins (data not shown). Thus, the interaction between BRCA1 and MLH1 may be mediated indirectly through other proteins. Consistent with this, a mass-spectrometry study showed that BRCA1 and other proteins, including MSH2, MSH6, MLH1, ATM, BLM, RAD50, MRE11, NBS and RFC, form a complex called BRCA1-associated genome-surveillance complex (BASC) (Wang et al., 2000) that is postulated to be involved in the recognition and repair of aberrant DNA structures. Wang et al. (2000) were able to demonstrate binding of BRCA1 to MSH2 and MSH6, both factors that bind directly to MLH1.

A candidate approach was used to study the localization and expression of several genes and gene products that are involved in various aspects of DNA-damage repair. We showed that distribution of Rad51 on chromosomes was disrupted in testes from Brca1 mutant mice. This finding is significant because Rad51 is known to interact with Brca1 either directly or indirectly after treatment with DNA-damaging reagents (Huber et al., 2001; Scully et al., 1997). Therefore, the abnormal distribution of Rad51 in mutant chromosomes provides strong supporting evidence for the hypothesis that full-length BRCA1 is required for the repair of DNA during meiosis in spermatocytes. Notably, the localization of Dmc1, a closely related partner of Rad51 (Masson and West, 2001; Tarsounas et al., 1999), was not altered, which indicates that loss of full-length Brca1 disrupts the function of some but not all DNA-damage-repair proteins.

Recent investigations have highlighted the interaction between γH2AX and BRCA1 and the importance of γH2AX in DNA-damage repair. After using a laser beam to generate a DSB, γH2AX becomes phosphorylated and moves to the site of the lesion within minutes (Paull et al., 2000). BRCA1 moves to the same site approximately 45 minutes later. This suggests that γH2AX serves as a DNA damage sensor and that it is responsible for recruiting BRCA1 to sites of DNA damage. In fact, BRCA1 foci do not form on chromosomes from mice that lack H2AX, which results in increased genetic instability (Celeste et al., 2002).

At the late zygotene and early pachytene stages of normal spermatogenesis, γH2AX relocates from sites of DSBs on chromosomes to the XY body. This did not occur in the absence of full-length Brca1. Instead, γH2AX remained as multiple, distinct foci on chromosomes. Because γH2AX binds to DNA prior to binding Brca1 (Paull et al., 2000), it is likely that the binding of γH2AX to DSBs is independent of Brca1 and that the absence of full-length Brca1 did not alter this process in spermatocytes. However, loss of full-length Brca1 did block relocation of γH2AX to the XY body. There are two possible explanations for this observation. First, recruitment of Brca1 by γH2AX may be essential for repairing DNA damage in spermatocytes. Therefore, in the absence of full-length Brca1, unrepaired DNA damage accumulates in the spermatocytes, which traps γH2AX and prevents it from moving to the XY bodies. Experimental support for this possibility comes from the observation that the phenotypes of control spermatocytes that sustained extensive DNA damage from γ-irradiation and that of Brca1 mutant spermatocytes were similar. Alternatively, Brca1 could play a role that actively recruits γH2AX to the XY body. The absence of Brca1 consequently results in the failure of γH2AX localization to the XY body. Recently, it has been proposed that Brca1 is involved in X-chromosome inactivation (Ganesan et al., 2002). Future studies are needed to determine whether loss of localization of γH2AX to the XY body in Brca1 mutant spermatocytes affects X-inactivation.

Although BRCA1 appears to regulate the expression of many genes that are involved in multiple biological processes (Chen et al., 1999; Deng and Brodie, 2000), only three of these genes are known to play a role in DNA-damage repair. These are Gadd45 (a DNA damage-repair and response gene) (Harkin et al., 1999), Ddb2 (which is defective in Xeroderma Pigmentosum group E cells and encodes the p48-damaged DNA-binding protein) (Takimoto et al., 2002) and Xpc (a gene encoding Xeroderma Pigmentosum group C complementing protein) (Hartman and Ford, 2002). All three genes are regulated by p53 and are involved in the nuclear excision-repair pathway (Hartman and Ford, 2002; MacLachlan et al., 2002; Takimoto et al., 2002). In this study, we found that loss of full-
length Brca1 correlated with decreased expression of several genes that encode proteins involved in the repair of DSBs, including RuvB-like DNA helicase, TFIIH (p62) and TFIIH. Additional investigations will determine if these genes are transcriptional targets of BRCA1 and if this regulation is direct or indirect.

The p53-dependent apoptotic pathway plays a dominant role in some but not all organs (reviewed by Burns and El-Deiry, 1999). We showed that spermatocyte apoptosis triggered by loss of full-length Brca1 was mediated by p53-dependent and p53-independent pathways. The most likely proximal trigger for both p53-dependent and p53-independent apoptosis in Brca1-deficient spermatocytes is the development of genetic instability. Thus, mutant spermatocytes that escaped the first wave of p53-dependent apoptosis survived slightly longer but still failed to progress into the diplotene stage. This suggests that activation of more than one apoptotic pathway ensures that spermatogenesis cannot be completed in the presence of significant genetic instability.

In summary, this study revealed that Brca1 plays an essential role in DNA-damage repair during spermatogenesis (Fig. 9). Brca1 functions through at least two different mechanisms, the recruitment of DNA damage-repair proteins to sites of DNA damage, and the regulation of the expression of DNA damage-repair genes. Examples of the first mechanism include the diminished numbers of Rad51 and Mlh1 foci on chromosomes in the absence of full-length Brca1. Examples of the second mechanism are the altered expression levels of RuvB-like DNA helicase, XPB, p62 and TFIID in the absence of full-length Brca1. Thus, Brca1-deficient spermatocytes unavoidably accumulate unrepaired DNA damage that triggers both p53-dependent and p53-independent apoptosis, and failure of spermatogenesis.

We thank Drs L. P. K. Jones, M. Tilli, R. H. Wang, L. Cao, W. Qiao, and other members of Dr Furth’s laboratory and Dr Deng’s laboratory for their helpful discussions.

REFERENCES


