

Centrosome Amplification and a Defective G₂–M Cell Cycle Checkpoint Induce Genetic Instability in BRCA1 Exon 11 Isoform–Deficient Cells

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Summary

Germline mutations of the *Brca1* tumor suppressor gene predispose women to breast and ovarian cancers. To study mechanisms underlying BRCA1-related tumorigenesis, we derived mouse embryonic fibroblast cells carrying a targeted deletion of exon 11 of the *Brca1* gene. We show that the mutant cells maintain an intact G₁–S cell cycle checkpoint and proliferate poorly. However, a defective G₂–M checkpoint in these cells is accompanied by extensive chromosomal abnormalities. Mutant fibroblasts contain multiple, functional centrosomes, which lead to unequal chromosome segregation, abnormal nuclear division, and aneuploidy. These data uncover an essential role of BRCA1 in maintaining genetic stability through the regulation of centrosome duplication and the G₂–M checkpoint and provide a molecular basis for the role of BRCA1 in tumorigenesis.

Introduction

Cell cycle checkpoints are essential for maintaining genetic stability (reviewed in Morgan and Kastan, 1997; Weinert, 1998). In response to DNA damage, mammalian cells arrest at different points in the cell cycle and initiate repair. For example, arrest in G₁ phase prevents damaged DNA from being duplicated, and arrest in G₂ avoids segregation of damaged chromosomes. Failures in cell cycle checkpoints can lead to the acquisition and accumulation of genetic alterations and karyotype abnormalities. These changes may result in the activation of oncogenes and/or the inactivation of tumor suppressor genes and ultimately result in tumorigenesis.

A putative role of the tumor suppressor gene *Brca1* in cell cycle checkpoints has been proposed (reviewed in Paterson, 1998). The *Brca1* transcripts are induced in

late G₁ and become maximal after the G₁–S checkpoint (Vaughn et al., 1996). BRCA1 proteins undergo hyperphosphorylation during late G₁ and S and are transiently dephosphorylated early after M phase (Chen et al., 1996; Ruffner and Verma, 1997). BRCA1 is also associated with many cell cycle proteins, including E2F, cdc2, and cyclins (Wang et al., 1997). In addition, overexpression of wild-type BRCA1 induces G₁–S arrest in tissue culture (Somasundaram et al., 1997), whereas expression of a mutant form attenuates the G₂–M checkpoint (Larson et al., 1997).

Functional analyses of the *Brca1* gene have been performed in mice by using gene targeting (Gowen et al., 1996; Hakem et al., 1996; Liu et al., 1996; Ludwig et al., 1997; Shen et al., 1998). Embryos homozygous for these mutations exhibit phenotypic variations and die at embryonic day 5.5 (E5.5)–E13.5. The early lethality associated with the loss of the BRCA1 poses a conundrum with respect to its involvement in mammalian development, tumorigenesis, and cell cycle regulation.

In this report, we studied functions of BRCA1 in cell cycle checkpoints using mouse embryonic fibroblast cells (MEFs), which carry a targeted deletion of exon 11 of the *Brca1* gene. Our data show that the mutant MEFs have an intact G₁–S checkpoint but are defective in a G₂–M checkpoint. Of note, about 25% of mutant cells also exhibit amplification of functional centrosomes, leading to the formation of multiple spindle poles within a single cell. These abnormalities directly result in the unequal segregation of chromosomes, abnormal nuclear division, and aneuploidy.

Results

Brca1^{Δ11/Δ11} Embryonic Fibroblast Cells Exhibited Proliferative Defects

Northern blot analysis detected two major transcripts of about 7.2 and 3.9 kb of the *Brca1* gene during embryonic development (Figure 1A). RT-PCR followed by sequencing indicated that the 3.9 kb transcript is a natural Δ11 product, which creates an in-frame fusion between exon 10 and exon 12 (Figure 1B). Using a Cre-loxP system, we specifically deleted exon 11 from the mouse germline and found that the resulting homozygous embryos (*Brca1*^{Δ11/Δ11}) expressed only the 3.9 kb transcript and died at E12.5–18.5 (X. X. et al., unpublished data). The 3.9 kb transcript present in the *Brca1* mutant cells was verified by RT-PCR and sequencing to be identical to the alternative splice form contained in the wild-type cells. This physiological splice variant of *Brca1* is apparently functional, since it significantly extends the lifespan of the mutant embryos compared to the *Brca1* nulls. This extended survival allows the assessment of BRCA1 function in MEFs.

We first studied the growth properties of MEFs derived from E14.5–16.5 wild-type, *Brca1*^{+Δ11}, and *Brca1*^{Δ11/Δ11} embryos. Compared with wild-type and heterozygous cells, mutant cells required more time to reach confluence, even at passage 1. When cells were plated at

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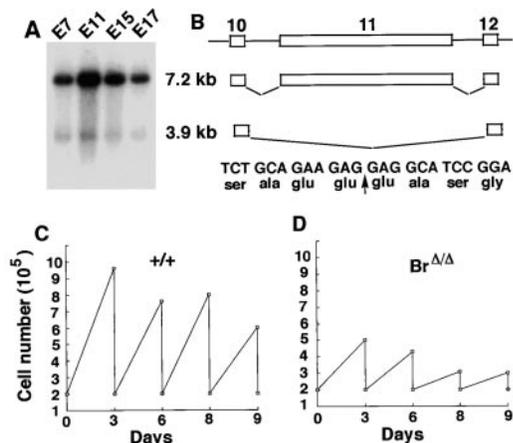


Figure 1. Targeted Deletion of Exon 11 of the *Brca1* Gene Results in Proliferative Defects

(A) Northern blot analysis of wild-type embryos, showing that the *Brca1* gene encodes two major transcripts of approximately 7.2 and 3.9 kb. A cDNA probe containing exons 10, 12, and 13 of the *Brca1* gene was used for hybridization.

(B) RT-PCR using primers located in exons 10 and 12 (not shown) followed by sequencing showed that the 3.9 kb transcript is a $\Delta 11$ product, which generates an in-frame fusion between exon 10 and exon 12 (arrow indicates the fusion junction).

(C and D) Representative growth curves of MEFs from E14.5 wild-type (C), and mutant (D) embryos. MEFs were plated at a density of 200,000 cells/well in 6-well plates. Every 3 days, cells were trypsinized, counted, and plated again at the same density. Three wild-type and three mutant MEF strains were studied and similar results were obtained.

equal density and counted at later time points, significantly fewer mutant cells were obtained than control cells (Figures 1C and 1D). These phenomena became progressively more pronounced at higher passage numbers. Theoretically, the lower proliferation rate of the mutant cells could be caused by increased cell death, slower progression through the cell cycle, or a combination of the two. To distinguish these, we carried out apoptosis and cell cycle analyses. TUNEL assays revealed no significant difference in cell death between wild-type and mutant cells. However, the rate of progression through the cell cycle, as determined by flow cytometry, was significantly slower in mutant MEFs compared to controls (data not shown and see below). These observations indicate that *Brca1* $^{\Delta 11/\Delta 11}$ cells have a prolonged cell cycle.

We next treated wild-type and mutant cells with genotoxins. Both control and mutant cells were found to be equally sensitive to mitomycin C and colcemid. However, a significantly increased sensitivity of mutant cells to γ radiation was observed (data not shown), suggesting a defect in DNA double-strand break repair.

Targeted Deletion of *Brca1* Exon 11 Causes Genetic Instability

BRCA1 has been implicated in DNA damage repair (Scully et al., 1997a, 1997b; Gowen et al., 1998). The repair function may be carried out by the BRCA1 11-containing isoforms, since BRCA1 interacts with RAD51 through a domain encoded by exon 11 (Scully et al.,

1997a). If the deletion of this exon results in an accumulation of unrepaired DNA damage, we would expect to see chromosomal aberrations. In order to comprehensively test this hypothesis, the karyotype of *Brca1* $^{\Delta 11/\Delta 11}$ MEFs was characterized by spectral karyotyping analysis (SKY) (Liyanage et al., 1996). At passage 1, 4 of 11 metaphases contained structural aberrations, including translocations and deletions. Evidence of chromosome breakage was seen in two metaphases. A representative cell is shown in Figures 2A–2C. This metaphase contains an acentric fragment (signaling chromosome breakage events) composed of segments from chromosomes 6 and 10, a translocation (4;7) and a deletion (7) as a result of an unbalanced translocation. Spontaneous chromosomal aberrations are characteristic of genetic instability; therefore, we examined cells from passage 3 for evidence of increasing chromosome abnormalities. Eleven of eleven metaphases analyzed by SKY contained structurally abnormal chromosomes. Evidence of chromosome breakage included quadriradial structures, chromatid gaps and deletions, acentric fragments, and complex rearrangements (Figures 2D and 2E). In some cells, double-minute chromosomes were also observed, indicating gene amplification (Figure 2E). Consistent with the increasing number of nonclonal chromosome aberrations during the first three passages, chromosome aneuploidy also became progressively pronounced in the mutant cells during the same period. About 40% of mutant cells were aneuploid at passage 1, with chromosome numbers ranging from 30 to 200 per cell, and the population of aneuploid cells increased to about 65% by passage 3. In contrast, less than 5% of aneuploid metaphases was observed in wild-type cells at both passage 1 and 3 (50 metaphases of wild-type and mutant MEFs were counted at each passage). These observations directly demonstrate that the loss of BRCA1 11 isoforms results in genetic instability.

Brca1 $^{\Delta 11/\Delta 11}$ MEFs Have an Intact G_1 -S Cell Cycle

Checkpoint but Are Defective in a G_2 -M Checkpoint
An essential function of cell cycle checkpoints is to prevent cells with damaged DNA from either replicating (G_1 -S checkpoint) or dividing (G_2 -M checkpoint). Cells derived from *Brca1* $^{\Delta 11/\Delta 11}$ embryos proliferate poorly in culture, suggesting that the loss of BRCA1 11 isoforms may activate one or more of these checkpoints. To test whether the loss of function of BRCA1 11 isoforms affected the G_1 -S checkpoint, we carried out cell cycle analysis on γ -irradiated cells by fluorescence-activated cell sorting (FACS). *p53* $^{-/-}$ cells were included as a control, since the G_1 -S checkpoint is dependent upon the p53 gene product (reviewed by Morgan and Kastan, 1997). As shown in Figures 3A and 3B, *p53* $^{-/-}$ cells exhibited very little reduction in the percentage of S phase cells relative to unirradiated samples after γ irradiation. In contrast, both wild-type and *Brca1* $^{\Delta 11/\Delta 11}$ cells displayed an approximate 50% reduction in the S phase cells. These observations indicate that the *Brca1* $^{\Delta 11/\Delta 11}$ cells have an intact G_1 -S checkpoint in response to γ irradiation.

Another DNA damage-responsive checkpoint is present at the G_2 -M transition, which rapidly delays movement of G_2 cells into mitosis (M) phase after γ irradiation.

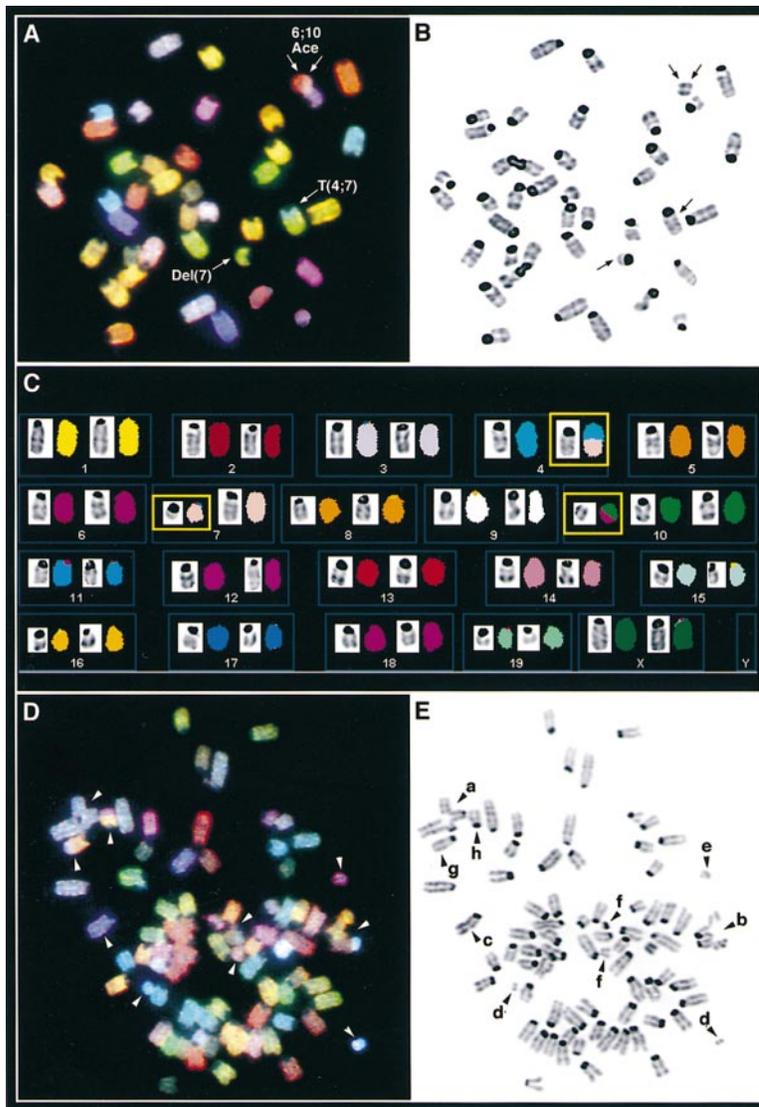


Figure 2. SKY Reveals Chromosomal Aberrations in *Brca1*^{Δ11/Δ11} MEFs

(A–C) SKY analysis was performed on passage 1 mutant MEFs. The hybridization is shown in display colors after hybridization in (A) and after DAPI banding (B). Display colors are generated by assigning red, green, or blue to specific spectral ranges. The arrows indicate chromosome aberrations, which are also highlighted in yellow boxes in the karyotype table (C). The spectra-specific classification, shown as pseudocolored chromosomes in (C), identifies the aberrations as a T (4;7), a Del (7), and an acentric fragment (Ace) containing segments from chromosomes 6 and 10.

(D–E) Analysis of a passage 3 tetraploid *Brca1*^{Δ11/Δ11} MEF metaphase. The display color image is shown in (D) and the inverted DAPI in (E). Lettered arrows indicate the following structural aberrations: (a) quadriradial (involvement of four chromatid arms, in this case all from chromosome 1), (b) complex rearrangement (more than four chromatid arms, in this case from chromosomes 3 (yellow), 10 (red), and Y (blue)), (c) chromatid gap (X chromosome), (d) double minutes (Y chromosome), (e) acentric fragment (chromosome 12), (f) a chromosome break in chromosome 6 (arrows indicate the separated centromere and chromosome arms), (g) T (17;6), and (h) T (14;12).

To see if the loss of BRCA1 11 isoforms affected this G₂–M checkpoint, cells were irradiated at 3 Gy, and the mitotic index (MI) was scored at various times postirradiation. A sharp reduction in MI was observed in both wild-type and p53^{-/-} cells within 1 hr after irradiation. In contrast, *Brca1*^{Δ11/Δ11} cells showed no reduction in MI over the 4 hr time course (Figure 3C). In subsequent experiments, cells were irradiated at increasing doses from 0.5 to 10 Gy. Wild-type and p53^{-/-} cells exhibited a dose-dependent reduction in MI, whereas *Brca1* mutant cells showed little reduction regardless of dose (Figure 3D). The lack of an immediate mitotic delay following γ irradiation indicates that elimination of BRCA1 11 isoforms abolishes this checkpoint and allows cells with damaged DNA to proceed into mitosis.

We next treated cells with UV, an inducer of pyrimidine dimers, or MMS (methyl methanesulfonate), a methylating agent. Notably, UV-treated *Brca1*^{Δ11/Δ11} cells showed a dramatic reduction in MI, while the MMS-treated mutant cells exhibited only a slight reduction relative to untreated controls (data not shown). Taken together,

these observations suggest that the defect in the G₂–M checkpoint is specific to certain types of DNA damage.

Centrosome Amplification and Unequal Chromosome Segregation in *Brca1*^{Δ11/Δ11} MEFs

The presence of a substantial percentage of aneuploid cells suggests that the fidelity of chromosome segregation is compromised. Recent studies have suggested that aberrant replication of centrosomes can result in defective mitotic spindle organization and lead to aneuploidy (Fukasawa et al., 1996; Pihan et al., 1998). To examine whether loss of BRCA1 11 isoforms affects centrosome duplication, centrosomes in first passage *Brca1*^{Δ11/Δ11} and control MEFs were detected with an antibody to γ -tubulin. One or two centrosomes were detected in wild-type cells at interphase and at various stages of M phase (Table 1, Figures 4A and 4B). In contrast, about 25% of *Brca1*^{Δ11/Δ11} cells contained more than two centrosomes in both interphase and M phase (Table 1, Figures 4C and 4D). In the most extreme case, a mutant cell was found containing 25 centrosomes

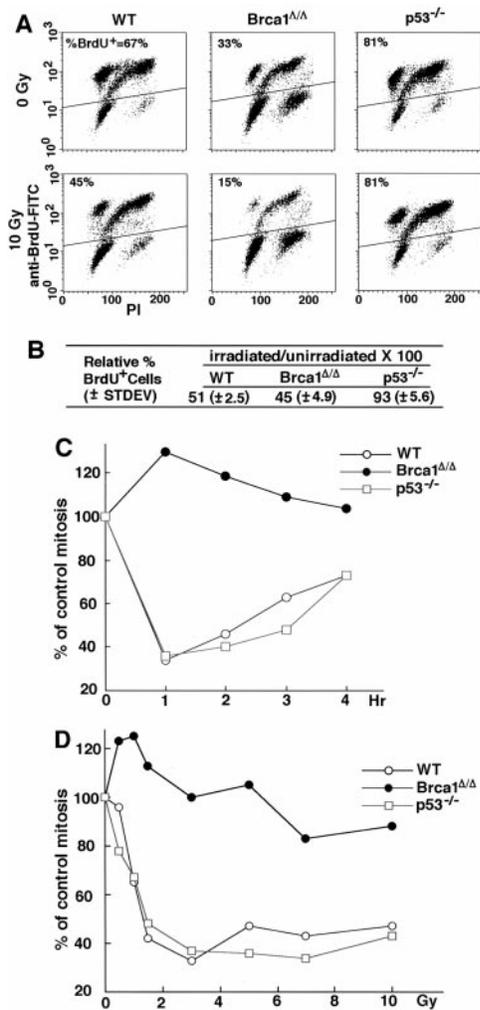


Figure 3. Intact G₁-S but Defective G₂-M Checkpoint in *Brca1*^{Δ11/Δ11} MEFs

(A) Representative FACS dot plots of synchronized wild-type (WT), *Brca1*^{Δ11/Δ11}, and *p53*^{-/-} MEFs 24 hr after exposure to 0 or 10 Gy γ radiation. For each analysis, 10,000 cells for each genotype were analyzed.

(B) Relative percent of BrdU⁺ cells. Percentages of BrdU⁺ cells were determined by FACS (n = 2) and fluorescence microscopy (n = 1 for *p53*^{-/-} cells and n = 2 for WT and *Brca1*^{Δ11/Δ11} cells) STDEV: standard deviation.

(C and D) Lack of mitotic delay in *Brca1*^{Δ11/Δ11} cells. Mitotic cells were counted 1–4 hr after treatment with 3 Gy γ radiation (C), or at 1 hr with doses of 0.5–10 Gy (D). Mitotic indexes of untreated cells were used as controls. Over 6,000 cells were counted at each dose. Three wild-type, three *Brca1*^{Δ11/Δ11}, and two *p53*^{-/-} MEF strains were studied and similar results were obtained. (C) and (D) show results from one representative experiment.

(data not shown). Since *p53*-deficient cells have been shown to contain increased numbers of centrosomes (Fukasawa et al., 1996), we included *p53*^{-/-} MEFs as a positive control in the immunostaining. The percentage of *p53*^{-/-} cells containing more than two centrosomes was slightly lower than that of *Brca1*^{Δ11/Δ11} MEFs under our conditions (Table 1).

Multiple centrosomes in the *Brca1*^{Δ11/Δ11} MEFs may

nucleate multipolar spindles, which could result in unequal chromosome segregation. Therefore, we examined whether cells with amplified centrosomes were associated with defects in mitotic spindle organization. Double immunostaining with γ -tubulin and α -tubulin (a component of microtubules) revealed that the additional centrosomes were frequently associated with spindles, resulting in the formation of multiple poles (Figure 4E). Consequently, many of the mutant cells in mitosis segregated their chromosomes abnormally (Figure 4F). This missegregation can cause unequal nuclear division, leading to the formation of multiple nuclei within a single cell (Figure 4G).

Discussion

We have assessed the genetic stability and cell cycle checkpoint functions of exon 11-containing isoforms of BRCA1 in MEFs by selectively deleting exon 11 of the gene. Our data indicate that *Brca1*^{Δ11/Δ11} cells are completely defective in a G₂-M checkpoint, which allows these cells to enter M phase with abnormal chromosomes. However, *Brca1*^{Δ11/Δ11} cells do have an intact γ radiation-induced G₁-S checkpoint, which may be responsible for the reduced proliferation observed in mutant cells. We also showed that *Brca1*^{Δ11/Δ11} cells have multiple copies of centrosomes and form abnormal mitotic spindles, resulting in aberrant chromosome segregation. These data provide molecular clues to the role of BRCA1 in maintaining genetic stability and inhibiting tumorigenesis.

BRCA1 and Genetic Stability

We postulate that the genetic instability observed in *Brca1*^{Δ11/Δ11} cells is due to a combination of defects in DNA damage repair, loss of a G₂-M checkpoint, and centrosome amplification. The aberrant chromosome segregation reported here is consistent with previous studies in human tumor cells. A cell line derived from a homozygous BRCA1-deficient breast tumor exhibits a high degree of aneuploidy (Tomlinson et al., 1998), and human breast tumors with mutations in the *Brca1* gene show a higher number of chromosomal gains and losses than tumors without BRCA1 mutations (Tirkkonen et al., 1997). We previously showed that mouse embryos with a loss-of-function mutation in BRCA1 exhibit chromosome abnormalities (Shen et al., 1998). Here, we show that metaphases of *Brca1*^{Δ11/Δ11} MEFs exhibit nonclonal accumulations of chromosome aberrations, including chromosome and chromatid breakage. Such aberrations are also found in chromosome instability syndromes like Fanconi anemia, ataxia telangiectasia, Bloom syndrome, Werner syndrome, and Nijmegen breakage syndrome (reviewed in Meyn, 1997). The potential relationship between *Brca1* and the genes that are responsible for these diseases needs to be determined.

Evidence that BRCA1 is involved in DNA damage repair comes from observations that BRCA1 colocalizes with RAD51 (Scully et al., 1997a) and is relocated to

Table 1. Number of Centrosomes in MEFs at Passage 1

No. Centrosome	Interphase			Mitosis		
	n = 1 or 2	n ≥ 3	Ab (%)	n = 1 or 2	n ≥ 3	Ab (%)
<i>Brca1</i> ^{+/+}	376	10	2.6	206	7	3.2
<i>Brca1</i> ^{Δ11/Δ11}	1005	241	24	259	83	27
<i>P53</i> ^{-/-}	308	60	16.3	236	65	22

intranuclear structures where DNA replicates after treatment with DNA-damaging reagents (Scully et al., 1997b). RAD51, a homolog of the yeast RecA protein, is involved in ATP-dependent DNA strand exchange reactions (Ogawa et al., 1993). Loss of BRCA1 may also result in deficiency in transcription-coupled repair (Gowen et al., 1998). Notably, embryos carrying a targeted disruption of RAD51, BRCA1, or BRCA2 share similar phenotypes. They are all hypersensitive to γ irradiation and exhibit early embryonic lethality, which is partially rescued by a p53 mutation (Lim and Hasty, 1996; Ludwig et al., 1997; Sharan et al., 1997; Shen et al., 1998). Moreover, studies on MEFs containing attenuation mutations of *Brca1* or *Brca2* indicate that both genes are essential for genetic stability (Patel et al., 1998; this study). These

striking similarities of *Rad51*, *Brca1*, and *Brca2* mutants suggest a functional link of these genes in DNA damage repair. Disruption of the proposed BRCA1/BRCA2/RAD51 complex may lead to genetic instability (Brugarolas and Jacks, 1997; Chen et al., 1998).

BRCA1 and Cell Cycle Checkpoints

Our data show that BRCA1 is not just a DNA repair protein, but that it is also involved in cell cycle control. To demonstrate this, we examined the status of several checkpoints in *Brca1*^{Δ11/Δ11} MEFs. Our data show that BRCA1 11 isoforms are not involved in the G₁-S checkpoint, since mutant cells showed G₀/G₁ arrest upon γ irradiation similar to that of wild-type controls. The intact G₁ checkpoint may be responsible for the proliferation

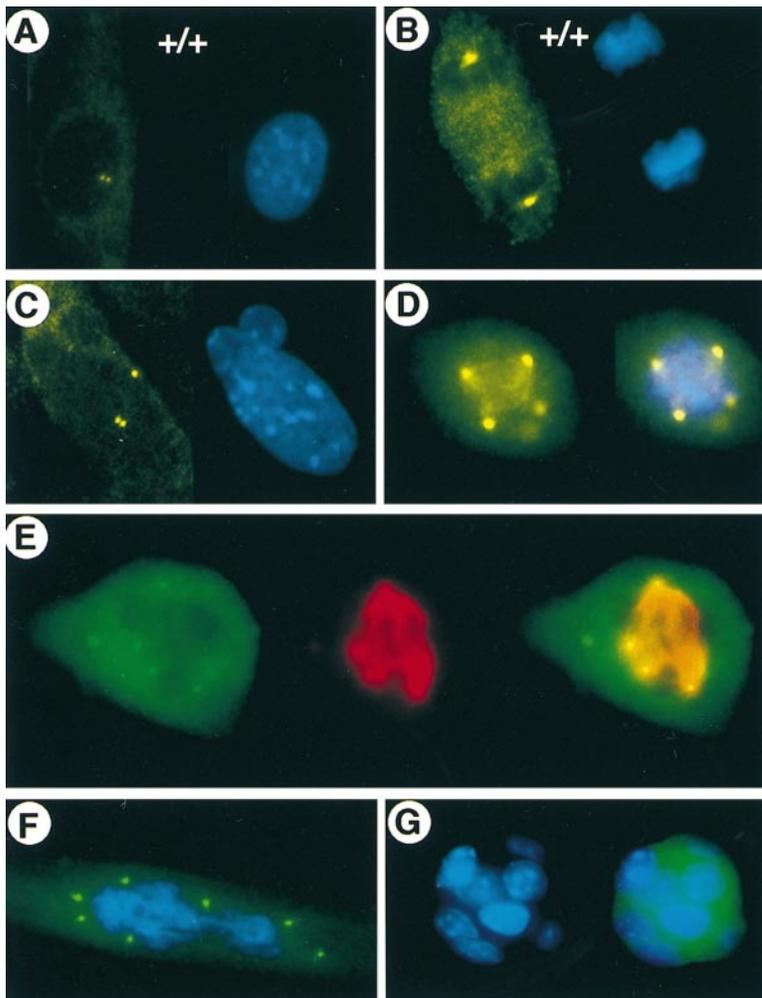


Figure 4. Centriole Amplification in *Brca1*^{Δ11/Δ11} MEFs

(A-D) MEFs were immunostained with anti- γ -tubulin (green) and DAPI-stained for DNA (blue). *Brca1*^{+/+} interphase (A) and mitotic (B) cells, both containing two centrosomes. (C-G) *Brca1*^{Δ11/Δ11} MEFs are shown. (C) Interphase cell with three centrosomes. (D) Mitotic cell displaying four centrosomes. (E) Double immunostaining with anti- γ -tubulin, and anti- α -tubulin (red) reveals that amplified centrosomes can nucleate spindle poles. (F) A mitotic cell with seven centrosomes and unequal segregation of genetic material. (G) An aberrant cell with multiple nuclei as a result of unequal nuclear division. All cells are shown at the same scale.

defects observed in the mutant cells. Since the G₁-S damage checkpoint is primarily controlled by the p53 tumor suppressor (reviewed by Morgan and Kastan, 1997), the presence of an intact G₁-S checkpoint in *Brca1* mutant cells suggests that the p53 pathway is functional. Indeed, preliminary data indicate that elimination of p53 significantly extends the survival of the *Brca1*^{Δ11/Δ11} embryos (X. X. et al., unpublished data). This is consistent with previous investigations showing that removal of p53 partially rescued the deficiency caused by BRCA1-null mutations (Hakem et al., 1997; Ludwig et al., 1997; Shen et al., 1998).

Interestingly, we found that the *Brca1*^{Δ11/Δ11} MEFs are defective in a γ irradiation-induced G₂-M checkpoint. Loss of this G₂-M checkpoint allows cells with damaged DNA to enter M phase, increasing the likelihood that abnormal chromosomes will be passed to the daughter cells. Since the p53-dependent G₁-S checkpoint is intact in these cells, the aberrations do not become clonal. Our data show that the *p53* and *Brca1* genes have very distinct functions in the G₁-S and G₂-M checkpoints. *Brca1*^{Δ11/Δ11} cells have an intact G₁-S checkpoint but are completely defective in the G₂-M checkpoint, whereas an opposite pattern is observed in p53^{-/-} cells.

BRCA1 and Centrosome Amplification

A novel finding in this study is that *Brca1*^{Δ11/Δ11} cells contain abnormal numbers of centrosomes. Centrosomes normally duplicate only once during each cell cycle. The duplication begins near the G₁-S boundary and is completed in G₂ phase. The duplicated centrosomes then move to opposite sides of the nucleus in M phase and organize the mitotic spindle apparatus. Two centrosomes per cell ensure the formation of a bipolar spindle, which is essential for the equal segregation of chromosomes (Rudner and Murray, 1996; Winey, 1996). BRCA1 appears to play an essential role in the regulation of centrosome duplication. In addition to the significant percentage of mutant cells that contained multiple centrosomes, we found that the centrosome duplication process is initiated earlier in *Brca1*^{Δ11/Δ11} cells than in normal cells (data not shown). BRCA1 11 isoform deficiency therefore leads to abnormal mitoses. Many human tumors, including high-grade breast tumors, contain abnormal centrosomes (Lingle et al., 1998; Pihan et al., 1998). By growing the mutant cells in culture, we were able to directly observe centrosome amplification, abnormal spindle formation, and unequal segregation of chromosomes, thus providing a direct link of the *Brca1*^{Δ11/Δ11} phenotype to a cancer-related defect. It was recently shown that BRCA1 is physically associated with the centrosome (Hsu and White, 1998). Considering that BRCA1 has both protein-protein interaction and transactivation activities (Lane et al., 1995; Chapman and Verma, 1996; Wu et al., 1996), it is possible that BRCA1 regulates the centrosome duplication process through transactivation of centrosome-specific genes.

It has been proposed that the *Brca1* tumor suppressor gene is in a class of caretaker genes, whose mutation does not directly promote tumor formation (Kinzler and Vogelstein, 1997). Indeed, somatic mutations of *Brca1* are rarely detected in sporadic tumors, and people carrying *Brca1* mutations develop tumors with a relatively

long latency, during which multiple events, including the loss of tumor suppressor genes and the activation of oncogenes may occur (Crook et al., 1997; Struwing et al., 1997; Tseng et al., 1997). Our finding that *Brca1*^{Δ11/Δ11} cells are genetically unstable provides genetic and functional evidence of the importance of BRCA1 in maintaining the stability of the genome.

Experimental Procedures

Cell Culture, Cell Cycle Analysis, and Spectral Karyotyping

Primary MEFs were obtained from E14.5-16.5 embryos that were either wild-type, *Brca1*^{Δ11/Δ11}, *Brca1*^{+Δ11}, or *p53*^{-/-} using a standard procedure. G₁-S checkpoint analysis was carried out as described previously (Deng et al., 1995; Linke et al., 1997). For G₂-M checkpoint analysis, cells in logarithmic growth were mock treated or irradiated with 1-10 Gy of γ radiation and then returned to their incubator. After 1-4 hr, cells were fixed with 2.5% paraformaldehyde in 25 mM MgCl₂/PBS for 30 min., washed with PBS, and then stained with DAPI. Mitotic cells in prophase, metaphase, anaphase, and telophase were identified by fluorescence microscopy and expressed as a fraction of the total. Spectral karyotyping of the embryonic cells was performed as described previously (Liyanage et al., 1996).

Centrosome Staining and Analysis

Cells grown on chamber slides (Falcon) were fixed in 2.5% paraformaldehyde plus 25 mM MgCl₂/PBS for 10 min room temperature, washed in 0.3 M glycine/PBS, permeabilized in 0.2% Triton X-100/PBS, and incubated overnight with polyclonal anti- γ -tubulin (Sigma) diluted 1:1000 in 5% goat serum/PBS. The antibody complexes were detected with FITC-conjugated goat anti-rabbit IgG (Boehringer Mannheim) and stained with DAPI. For dual detection of centrosomes and microtubules, cells were fixed in ice-cold methanol for 10 min, and the permeabilization step was eliminated. Immunostaining was performed in four layers: anti- γ -tubulin followed by Texas red-conjugated goat anti-rabbit IgG (Vector Laboratories), and then anti- α -tubulin (Sigma), diluted 1:500 in 5% goat serum/PBS followed by FITC-conjugated sheep anti-mouse IgG (Boehringer Mannheim). Gray level images were acquired using a CCD camera (CH250, Photometrics, Tucson, AZ) mounted on a Leica DMRBE epifluorescence microscope, and pseudocolored using Registration software.

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