Extensive Chromosomal Instability in Rad51d-Deficient Mouse Cells

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Abstract
Homologous recombination is a double-strand break repair pathway required for resistance to DNA damage and maintaining genomic integrity. In mitotically dividing vertebrate cells, the primary proteins involved in homologous recombination repair are RAD51 and the five RAD51 paralogs, RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3. In the absence of Rad51d, human and mouse cells fail to proliferate, and mice defective for Rad51d die before birth, likely as a result of genomic instability and p53 activation. Here, we report that a p53 deletion is sufficient to extend the life span of Rad51d-deficient embryos by up to 6 days and rescue the cell lethal phenotype. The Rad51d<sup>−/−</sup> Trp53<sup>−/−</sup> mouse embryo-derived fibroblasts were sensitive to DNA-damaging agents, particularly interstrand cross-links, and exhibited extensive chromosome instability including aneuploidy, chromosome fragments, deletions, and complex rearrangements. Additionally, loss of Rad51d resulted in increased centrosome fragmentation and reduced levels of radiation-induced RAD51-focus formation. Spontaneous frequencies of sister chromatid exchange were not affected by the absence of Rad51d, but sister chromatid exchange frequencies did fail to be induced upon challenge with the DNA cross-linking agent mitomycin C. These findings support a crucial role for mammalian RAD51D in normal development, recombination, and maintaining mammalian genome stability. (Cancer Res 2005; 65(6): 2089-96)

Introduction
Genetic defects in DNA repair genes are likely responsible for chromosome rearrangements associated with the evolution of cancer (1). One type of DNA damage, the double-strand break (DSB), is formed during DNA replication, antigen receptor rearrangements, or upon exposure to environmental or cancer therapy agents. Conventional two-ended DSBs are repaired by nonhomologous end joining or homologous recombination (HR) repair pathways, and defects in either pathway lead to a failure to properly repair damaged DNA which contributes to chromosome unstable phenotypes (2).

HR is primarily directed by the RAD51 recombination/repair family (3, 4). Altered expression and mutations in RAD51 genes are associated with chromosome rearrangements in human cancers and selected resistance to chemotherapeutic agents (1, 5–7). The RAD51D protein is ~39% similar in sequence and structure to RAD51 and contains two conserved Walker box motifs that predicted binding and hydrolysis of ATP (8–11). Biochemical analysis of RAD51D showed DNA-stimulated ATPase activity independently and in complex with XRCC2 (12, 13). RAD51D/XRCC2 heterodimers have the ability to form filamentous structures along ssDNA that promote homologous pairing between ssDNA and dsDNA molecules (14), and the RAD51D/XRCC2 complex interacts with the NH2-terminal domain of the BLM helicase protein to stimulate resolution of synthetic Holliday junctions (13). These data suggest RAD51D is involved in at least two aspects of HR, strand invasion and Holliday junction resolution.

Mice carrying a homozygous targeted deletion in the Rad51d gene die during embryo development, surviving up to 10.5 days post-conception. Mutant embryos have a range of phenotypes and increased levels of cell death, suggestive of random mutations in cell and developmental regulatory genes (15). Supporting a role for RAD51D in genome maintenance in vertebrates, chicken B lymphocyte DT40 cell lines carrying a rad51d disruption accumulated chromosomal breaks and had increased levels of dead cells (16). Similarly, when RAD51D synthesis was inhibited by small interfering RNA in human cells, cultures died within 7 days (17). Here, we report on mice and isogenic mouse embryo fibroblasts (MEF) deficient for both Rad51d and Trp53. Double mutant embryos survive up to 16.5 days post-conception and unlike Rad51d<sup>−/−</sup> MEFs, Rad51d<sup>−/−</sup> Trp53<sup>−/−</sup> MEFs proliferate in culture. By generating viable Rad51d<sup>−/−</sup> Trp53<sup>−/−</sup> cell lines, we, for the first time, have been able to investigate cellular phenotypes conferred by a Rad51d disruption in mammalian cell lines and perform detailed analyses of chromosomal instability in primary, nonimmortalized, Rad51d-deficient cells by multiple methods, including spectral karyotype analysis. Mutant cells had elevated levels of chromosome instability, increased centrosome fragmentation, were hypersensitive to DNA damaging agents, and failed to form radiation-induced RAD51 foci. These results show that RAD51D plays a critical role in maintaining the integrity of the mammalian genome and loss of p53 is sufficient for proliferation of these highly compromised and chromosomally unstable cells.

Materials and Methods

Breeding Scheme and Genotyping. Mice heterozygous for Rad51d (15) and Trp53 (18), obtained from The Jackson Laboratory (Bar Harbor, ME), were crossed to generate mice heterozygous for both genes. Both lines of mice were in a C57BL6/J strain background. Following timed matings, embryos from E9.5 to E16.5 were dissected and either fixed in 10% formalin or homogenized for generating cell lines. Rad51d-deficient cells by multiple methods, including spectral karyotype analysis. Mutant cells had elevated levels of chromosome instability, increased centrosome fragmentation, were hypersensitive to DNA damaging agents, and failed to form radiation-induced RAD51 foci. These results show that RAD51D plays a critical role in maintaining the integrity of the mammalian genome and loss of p53 is sufficient for proliferation of these highly compromised and chromosomally unstable cells.
Table 1. Genotypes of progeny from Rad51d−/− Trp53−/− intercrosses

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>Rad51d−/−</th>
<th>Rad51d+/+</th>
<th>Rad51d+/+ Trp53+/+</th>
<th>Resorbed embryos</th>
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<tr>
<td>E9.5</td>
<td>17</td>
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<td>E10.5</td>
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<td>3 (3.9)</td>
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<td>E12.5</td>
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<td>2 (5.1)</td>
<td>1</td>
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<tr>
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<td>2 (3.7)</td>
<td>14 (17.4)</td>
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<td>E14.5</td>
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<td>E16.5</td>
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<td>Live pups</td>
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<td>2 (6.0)</td>
<td>0 (28.5)*</td>
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</table>

NOTE: The Rad51d and Trp53 genes are 9.5cM apart on mouse chromosome 11, and the targeted gene disruptions are in the cis-formation. The expected number of embryos for the reported genotypes is shown in parentheses.

*The observed number of progeny is significantly different compared to the expected number of progeny from \( \chi^2 \) test, \( P < 0.05 \).

Cell Culture, Growth, and Chromosome Analysis. Primary cultures of E13.5 to E14.5 embryos were prepared by homogenizing whole embryos in DMEM supplemented with 7.5% fetal bovine serum, 7.5% NCS, and antibiotics, to single cell suspension and plating to a 60-mm dish. For cell growth analysis, MEFs were seeded onto 6-well plates at 8 \( \times 10^4 \) cells per well. Cells were counted using a Coulter counter (Coulter Beckman, Fullerton, CA). Flow cytometric analysis (Coulter Beckman) was carried out on two independent exponentially growing primary MEF cultures per genotype stained with propidium iodide.

Giems-stained metaphase chromosome spreads were prepared from primary MEF cells. Two independent MEF cell lines per genotype were scored for chromatid and chromosome abnormalities as described (ref. 2b; Rad51d+/- Trp53+/-, n = 206; Rad51d+/+ Trp53−/−, n = 200; Rad51d−/− Trp53−/−, n = 204 spreads). Spectral karyotype analysis from two independent primary MEF cell lines per genotype was done and scored by the Germline Modification Laboratory at the Van Andel Research Institute (Grand Rapids, MI) using Applied Spectral Imaging software (Rad51d−/− Trp53−/−, n = 21; Rad51d+/− Trp53−/−, n = 31; Rad51d−/− Trp53−/−, n = 13 spreads). For sister chromatid exchange (SCE) analysis, 3 \( \times 10^6 \) high passage (passage > 15) MEF cells were seeded onto 150-mm dishes and grown in McCoy’s 5A medium (Clontech, Palo Alto, CA). A full-length MmRad51d cDNA was cloned in frame into HA-pCMV5 solution (Clontech, Palo Alto, CA). Expression of Rad51d+/- /C0 and Rad51d−/− /C0 genes are 9.5cM apart on mouse chromosome 11, and the targeted gene disruptions are in the D37 value for the control ([B - A] / (C - A)] \times 100%, where A is the D37 value for mutant (Rad51d−/− Trp53−/−) cell lines, B is the D37 value for the complemented (Rad51d−/− Trp53−/−) cell lines, and C is the D37 value for the control (Trp53−/−) cell lines.

Immunofluorescence. Cells were seeded onto glass coverslips, grown to subconfluent levels, and, for the RAD51 foci experiments, treated with 10 Gy of irradiation. After 5 hours recovery, cells were fixed with 4% paraformaldehyde, permeabilized with a 0.3% Triton X-100 solution, blocked with 5% dry milk in 1xPBS, and incubated with a 1:100 dilution (diluted in block solution) of anti-HsRAD51 polyclonal antibody (Calbiochem, San Diego, CA) overnight at room temperature. Cells were then washed with 1x PBS and incubated with the Oregon Green 488 goat anti-rabbit IgG secondary (1:1,200; Molecular Probes, Eugene, OR, diluted in block solution) for 1 hour at room temperature. Cells were washed with 1x PBS, stained with 4’-diaminido-2-phenylindole dihydrochloride hydrate (Sigma; 200 ng/mL) for 10 minutes, coded, and mounted onto glass slides. Two methods were used to score RAD51 foci. Cells containing five or more distinct RAD51 foci were scored positive (untreated: Rad51d+/- Trp53−/−, n = 452; Trp53−/−, n = 812; Rad51d−/− Trp53−/−, n = 850 cells; treated with 10 Gy: Rad51d−/− Trp53−/−, n = 437; Trp53−/−, n = 756; Rad51d+/- Trp53−/−, n = 891 cells) and, in a separate analysis, the numbers of RAD51 foci per cell were scored (50 cells per genotype). For RAD51 protein expression analysis, whole cell MEF extracts were run on a 12% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were probed sites of pCDNA3.1 hygro+ (Invitrogen). High passage Rad51d−/− Trp53−/− MEFs were transfected using Lipofectamine Plus (Invitrogen) and selected with 250 μg/L hygromycin B. Resistant colonies were screened by Western blotting with 0.1 μL primary antibody raised against the HA epitope (12CA5; Roche, Indianapolis, IN). Two independent clones expressing detectable levels of HA-RAD51 protein were tested for MMC resistance. A polyclonal population of empty vector control cells was used as a control.
Characterization of Rad51d-Deficient Mouse Embryonic Fibroblast Cells. Rad51d is essential in the mouse for embryo and cell viability (15). Here, we report

phenotypes of Rad51d Trp53 double mutant embryos and embryo fibroblast cells, after performing timed matings of Rad51d−/− Trp53−/− heterozygous mice and embryo dissections. Lethality of Rad51d-deficient embryos was bypassed up to 6 days by deletion of the Trp53 gene (Table 1; the Rad51d and Trp53 genes are 9.5 cM apart on mouse chromosome 11, and for these experiments, the targeted gene disruptions were in the cis-formation). Mutant and wild-type embryos seem grossly similar at the midgestational stages, but by E13.5, Rad51d−/− Trp53−/− embryos are developmentally delayed and decreased in length by up to 50%. Double mutant embryos had nonconsistent phenotypes of varying

Figure 1. Characterization of Rad51d-deficient mouse embryos and MEF cell lines. A, representative normal and mutant mouse embryos. Rad51d−/− Trp53−/− embryos (left to right) with exencephaly (E14.5), craniofacial and spinal defects (E14.5), hemorrhaging (E15.5), and decreased size (E16.5). Bar, 5 mm. B, for genotyping, genomic DNA from primary MEF cell lines was digested with BamHI and analyzed by Southern blotting (probe B), as described (ref. 15; 20.0- and 8.6-kb fragments represent the disrupted and wild-type Rad51d alleles, respectively). The status of Rad51d was determined by PCR genotyping (600- and 450-bp fragments identify the disrupted and wild-type Trp53 alleles, respectively), and absence of Rad51d message confirmed by reverse transcription-PCR (RT-PCR). Primers specific to Rad51d cDNA amplify a 386-bp fragment (control primers were G3PDH, which amplify a 452-bp fragment). C, growth curves of primary MEF cells, homozygous wild-type (●), Rad51d−/− Trp53−/− (▲), and Rad51d−/− Trp53−/− (●). Points, two independent cell lines per genotype; bars, SE. D, representative FACs profiles from primary cells of the indicated genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spreads scored</th>
<th>Total chromosomes</th>
<th>Chromatid</th>
<th>Chromosome</th>
<th>Giant marker chromosomes</th>
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<td>Gaps</td>
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<tr>
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<td>9,063</td>
<td>9.93</td>
<td>21.0</td>
<td>7.72</td>
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<tr>
<td>Trp53+/−</td>
<td>200</td>
<td>9,511</td>
<td>6.31</td>
<td>11.6</td>
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</tr>
<tr>
<td>Rad51d+/− Trp53+/−</td>
<td>204</td>
<td>11,365</td>
<td>91.5†</td>
<td>77.4 †</td>
<td>132</td>
</tr>
</tbody>
</table>

*Data represent the total number of aberrations per chromosome × 10−4 from two independent cell lines for each genotype.

†Significant compared to wild-type and Trp53+/− cells at P < 0.05.

Results

Eliminating p53 Rescues the Lethality of Rad51d-Deficient Mouse Embryonic Fibroblast Cells. Rad51d is essential in the mouse for embryo and cell viability (15). Here, we report

Table 2. Chromosome aberrations in primary MEF cells

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<thead>
<tr>
<th>Genotype</th>
<th>Spreads scored</th>
<th>Total chromosomes</th>
<th>Chromatid</th>
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<td>11,365</td>
<td>91.5†</td>
<td>77.4 †</td>
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severity, suggestive of random mutations in developmental regulatory genes (Fig. 1A). Distinguishing features were blood islands, tissue necrosis, female specific exencephaly, and spinal and craniofacial malformations. To date, no Rad51d/−/− Trp53/−/− live pups have been identified and no increase in spontaneous tumors observed in >120 Rad51d+/− Trp53+/− mice up to 12 months of age.

Unlike mouse and human cells deficient for Rad51d (15, 17), Rad51d/−/− Trp53/−/− primary MEFs, isolated from E13.5- to E14.5 embryos, were able to proliferate in culture. To confirm the cells were derived from double mutant embryos, the genotypes and absence of Rad51d expression were verified following several passages (Fig. 1B). Primary double mutant cells had delayed growth rates (Fig. 1C), but no senescence or crises were observed, likely due to absence of p53 (23). Growth rates of mutant cells did increase to wild-type levels upon passaging (data not shown). Flow cytometric analysis of early passage mutant cells suggested increased cell death, as high levels of sub-G₁ signals are observed, and the percentage of S-G₂ and hyperploid cells is consistent with genome instability (Fig. 1D).

**Chromosome Instability in Rad51d-Deficient MEFs.** To assay for chromosome defects, Giemsa-stained metaphase spreads were scored from primary MEFs (Table 2). Rad51d-deficient cells had significantly increased levels of spontaneous chromatid breaks (11.3-fold) and gaps (4.7-fold), chromosome exchanges (24-fold), which likely result from inappropriate recombination, and end-to-end fusions (6.2-fold). Additionally, chromosomes at least twice the length of normal chromosomes, termed giant marker chromosomes, were observed at an increased frequency in the double mutants (9.2-fold). The frequencies of spontaneous chromosome gaps or breaks did not significantly increase in Rad51d-deficient cells. Consistent with the chromosome instability phenotype being directly related to loss of Rad51d, high passage Rad51d/−/− Trp53/−/− MEFs stably expressing a full-length, HA-Rad51d mouse cDNA had significantly lower levels of each chromosome abnormality compared with Rad51d/−/− Trp53/−/− cells transfected with an empty vector (data not shown).

To measure forms and frequencies of chromosomal rearrangements, spectral karyotype analysis was done (Fig. 2). A majority of identified abnormalities included nonreciprocally heterologous chromosome rearrangements, deletions, and fragments. The total number of chromosome aberrations in Rad51d/−/− Trp53/−/− cells was increased 39.7-fold and 12.1-fold compared with wild-type cells (0.0012 detached centromeres) and homoygous wild-type and Rad51d/−/− Trp53/−/−, P < 0.001; Trp53/−/− versus Rad51d/−/− Trp53/−/−, P < 0.001; Fig. 2B). Rad51d/−/− Trp53/−/− MEFs had a high frequency of detached centromeres (wild type, 0.0012 ± 0.0012; Rad51d/−/− Trp53/−/−, 0.0007 ± 0.0007; Rad51d/−/− Trp53/−/−, 0.0172 ± 0.0064 detached centromeres/chromosome) and increased levels of chromosome gain and loss (wild type, 0.762 ± 0.269; Trp53/−/−, 0.677 ± 0.329; Rad51d/−/− Trp53/−/−, 2.69 ± 0.936 chromosomes/spread).

**Decreased Levels of DNA Damage Induced SCE and RAD51 Focus Formation in Rad51d-Deficient Cells.** As a measure of HR, spontaneous and DNA damage induced SCE frequencies were determined (Fig. 2C). Because RAD51D is an HR protein shown to perform strand invasion (14) and rad51d/−/− DT40 cells have decreased SCE levels (16), we anticipated that Rad51d deficient MEFs would have a reduced SCE frequency. However, spontaneous SCE levels in Rad51d/−/− Trp53/−/− cells were not significantly different compared with Rad51d/−/− Trp53/−/− controls (Rad51d/−/− Trp53/−/− versus Rad51d/−/− Trp53/−/−, P = 0.979). Upon treatment with MMC, SCE frequencies increased ~3-fold in control Rad51d/−/− Trp53/−/− and Trp53/−/− cells, but failed to increase in Rad51d/−/− Trp53/−/− cells (Rad51d/−/− Trp53/−/−: treated versus untreated, P = 0.238). Although treating the mutant and control cells at different concentrations of MMC complicates the interpretation of the data, these results suggest that in mouse...
cells, RAD51D is not essential for the rescue of stalled replication forks during DNA replication (24), but is critical for DNA strand invasion of the homologous sister chromatid during the repair of interstrand cross-links (ICL).

To investigate whether RAD51D is necessary to recruit RAD51 to damaged DNA in mouse, MEFs were treated with X-rays and monitored for the presence of RAD51 foci (Fig. 3A). No significant difference was observed between nonirradiated Rad51d−/− Trp53+/− and Trp53−/− control cells (P = 0.172). However, there was a significant decrease in the percentage of nonirradiated Rad51d−/− Trp53+/− RAD51 foci–positive cells (Rad51d−/− Trp53+/− versus Rad51d−/− Trp53−/−, P < 0.001; Trp53−/− versus Rad51d−/− Trp53−/−, P < 0.001; Fig. 3B). Following treatment with 10 Gy of irradiation, the percentage of RAD51 foci–positive Rad51d−/− Trp53−/− cells failed to be induced to control levels (Rad51d−/− Trp53+/− versus Rad51d−/− Trp53−/−, P = 0.001; Trp53−/− versus Rad51d−/− Trp53−/−, P < 0.001). Western analysis confirmed protein levels of RAD51 were similar among all cell lines tested (data not shown). Similar results were obtained by scoring the number of RAD51 foci per cell (data not shown). These data support that the mouse RAD51D protein is necessary for the recruitment of RAD51 to DNA damage sites following exposure of mammalian cells to X-rays.

**Increased Levels of Centrosome Fragmentation in Rad51d-Deficient Cells.** MEF cell lines deficient for Xrcc2 and Chinese hamster ovary cell lines deficient for Xrcc2 or Xrcc3 have increased frequencies of centrosome fragmentation linked to chromosome nondisjunction (25, 26), but Rad51c defects did not influence formation of centrosomes (27). To examine whether loss of Rad51d affects centrosome stability, centrosome numbers were analyzed in early passage MEFs. A significant increase in the percentage of mitotic Rad51d−/− Trp53−/− cells having an abnormal number of centrosomes, compared with Trp53−/− cells (P = 0.003) and wild-type cells (P < 0.001), was observed (Fig. 3C). In contrast to the centrosome instability observed in Xrcc2−/− compared with wild-type MEFs (26), the frequency of abnormal centrosomes in Rad51d−/− Trp53−/− cells was not significantly different compared with wild-type controls (P = 0.900). These data suggest that genome instability in absence of RAD51D confers centrosome fragmentation, potentially through the process of mitotic catastrophe (28).

**Sensitivity to DNA-Damaging Agents.** The DNA repair capacity of high passage cell lines was assessed by colony survival assays following treatment with DNA damaging agents (Fig. 4). Rad51d−/− Trp53−/− cell lines were most sensitive to the DNA interstrand cross-linking agents, MMC (17.6-fold) and cisplatin (9.0-fold), and the DNA alkylating agent, methyl methanesulfonate (6.3-fold). The Rad51d-deficient cells were mildly sensitive to X-rays (1.5-fold), which induce DNA DSBs directly and indirectly by producing increased levels of free radicals, and UV light (1.4-fold), which can induce low amounts of ICL dimers (29) that may lead to double strand breaks during DNA replication (30). A full-length HA-Rad51d cDNA complemented the sensitivity phenotypes in Rad51d−/− Trp53−/− deficient cells for each DNA damaging agent tested, with the exception of the alkylating agent, methylmethane sulfonate. These data suggest that RAD51D plays a significant role in DNA cross-link repair and support that HR is the primary repair pathway of DNA ICLs (4). Additionally, there is no indication for haploinsufficiency for Rad51d.

**Discussion**

HR is critical for a number of biological systems; the generation of genetic diversity, growth and development, chromosome segregation during meiosis, and repair of complex forms of DNA damage. Recently, HR defects have been associated with cancer formation and progression (1, 5, 7). The data presented here show that loss of the HR gene Rad51d disrupted genome integrity in mouse cells, increasing levels of chromatid breaks and gaps,
together with the data presented here, suggest HR proteins play a more universal and vital role than NHEJ-associated proteins during high rates of cell proliferation, such as embryogenesis.

Recently, it was shown that RAD51D localizes to telomeres. Human and mouse cells lacking RAD51D have shorter telomeric DNA and an increased frequency of telomere fusions (17). In Rad51d-deficient mouse embryos, telomere dysfunction is likely one source for p53-mediated growth arrest (46). The increased frequency of chromosome fusions and giant marker chromosomes in Rad51d−/− Trp53−/− MEFs likely resulted, in part, from telomere fusions and chromosome translocations via the breakage-fusion-bridge cycle. Increased levels of telomere fusions were not observed in mouse fibroblasts deficient for Xrc2 (26). Similarly, in chromatin immunoprecipitation analyses, antibodies specific to RAD51D, but not RAD51 or the RAD51D interacting proteins RAD51C and XRCC2, pulled down significant levels of telomeric DNA (17). These data suggest that RAD51D is unique among the RAD51 family of proteins in maintaining genomic integrity by telomere protection.

Rad51d-deficient mice die at ~10.5 days post-conception (15), compared with xrc2-deficient mice, which can survive to birth (47). Despite both mutant MEF cells having increased levels of genome instability, Xrc2-deficient MEFS containing functional p53 could be immortalized whereas the rad51d-deficient MEFS failed to proliferate (26). Rad51d−/− Trp53−/− MEFS had similar phenotypes to xrc2-deficient MEFS and the rad51d-deficient chicken DT40 cell lines, including hypersensitivity to DNA cross-linking agents and ionizing radiation, failure to form radiation-induced RAD51 foci, and accumulated chromosomal breaks (16, 26). However, spontaneous SCE levels were not affected by loss of Rad51d in mouse cells, whereas the xrc2-deficient MEFS and rad51d mutant DT40 cells had a 1.4- and 1.9-fold decreased level of spontaneous SCEs, respectively. This suggests that mouse RAD51D is not essential to restart stalled DNA replication forks using the sister chromatid, but is critical for HR repair of ICLs using the sister chromatid as a replication template. Additionally, Rad51d-deficient MEFS and Xrc2-deficient MEFS were 17.6- and 4.5-fold more sensitive to the DNA cross-linking agent MMC, respectively, suggesting a more vital role for RAD51D in repairing ICLs (26).

In this study, a full-length mouse Rad51d cDNA complemented the cell sensitivity phenotypes of Rad51d−/− Trp53−/− MEFS to MMC, cisplatin, X-rays, and UV light but failed to complement sensitivity to the alkylating agent methylmethane sulfonate. We speculate that the engineered NH4-terminal epitope tag may be interfering with formation of recombinosome complexes specific to repairing methylmethane sulfonate–associated DNA lesions (8, 48). Alternatively, variant splicing (49) or regulated expression of Rad51d might be necessary for HR repair of alklylation DNA damage.

The increase in spontaneous chromosomal aberrations in Rad51d−/− Trp53−/− MEFS may result from a failure to resolve HR-associated DNA intermediates as opposed to defects in strand invasion. RAD51D is part of a protein complex with RAD51B, RAD51C, and XRCC2, termed BCDX2 (50–53). Interestingly, the recent implication of the BCDX2 complex in branch migration (54) and the direct interaction of RAD51D with the BLM helicase protein to stimulate the resolution of synthetic Holliday junctions (13), suggest a function for the RAD51 paralogues in resolving Holliday junction intermediates. We propose dual roles for RAD51D in both HR and telomere protection. For recombination, biochemical, and SCE data support RAD51D is involved in strand

aneuploidy, and chromosome rearrangements. Additionally, RAD51D was essential for repairing a number of DNA lesions, particularly ICLs.

Eliminating the Trp53 gene was sufficient to increase the life span of Rad51d-deficient embryos up to 6 days and rescue cell lethality, despite such extensive chromosome instability. Previous reports also describe partial or complete bypass of embryonic lethality by a Trp53 deficiency in mice carrying targeted disruptions in DSB repair genes (31–35). Mice deficient for the NHEJ genes, Xrcc4 or Lig4, die during late embryonic development (E16.5; refs. 36–38), and mutant phenotypes include increased neuronal cell death, impaired lymphocyte development, and growth deficiency. A Trp53 disruption rescued the neuronal cell death phenotype conferred by each gene disruption and completely rescued embryonic lethality (31, 32). In contrast, mice deficient for the HR genes Rad51 or Rad51b die during early gestation (E6.5–8.5; refs. 33, 34, 39), and when crossed into a Trp53−/− background, embryo lethality was bypassed at least 1 to 2 days (33, 34). The BRCA2 protein has roles in HR and interacts directly with RAD51 through BRC motifs (40–43). BRCA2 null mouse embryos die ~7.5 to 8.5 days post-conception (35, 44, 45) and, as observed in the Rad51 knockout mouse, embryo lethality was bypassed ~1 to 2 days by a Trp53-disruption (35). These results, together with the data presented here, suggest HR proteins play...
invasion during the processing of ICLs (14, 16) and in resolving recombination-associated DNA intermediates (13, 54). Because RAD51D is the only RAD51 paralog known to be present at telomeres (17), it may have a unique and independent role in the strand invasion step during formation of the protective T-loop structure, when the 3′ single-stranded G-rich overhang invades duplex telomeric DNA and pairs with the complementary C-rich strand (55). As an alternative or additional role, RAD51D may function in concert with the BLM protein at the telomere (56) to catalyze the resolution of the Holliday junction-like T loops, allowing for chromosome end replication.

In summary, these data show that RAD51D is critical for maintaining chromosome integrity in the mouse and for accurate repair of DSBs and ICLs. The absence of p53 is sufficient to bypass the cell lethal phenotype, permitting accumulation of complex genome rearrangements. These cell lines may help define mechanisms for tumor cells to continue proliferating although genomic integrity has been substantially compromised. Such chromosome instability and continued cellular proliferation is observed in Fanconi anemia, Bloom syndrome, ataxia telangiectasia, and solid tumors (5). Clinical data suggest that defects in the RAD51 paralogs contribute to the formation of breast, uterine, skin, or bladder cancer (1, 5, 7) and telomere attrition promotes epithelial cancers in p53-deficient mice (57). The RAD51-deficient MEF cell lines will be useful for structure and function analysis, determining RAD51D-dependent HR mechanisms, and may serve as a model for understanding the episodic genome instability phenomenon during early carcinogenesis. Such investigations will further define the function of RAD51D in maintaining chromosome stability, which may underlie protection against tumorgenesis.

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