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Loss of Brca2 and p53 Synergistically Promotes Genomic Instability and Deregulation of T-cell Apoptosis[1]

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ABSTRACT

***BRCA2* is a breast cancer susceptibility gene of which the product is thought to be involved in monitoring genome integrity and cell cycle progression. Brca2-null mice have a defect in embryonic cellular proliferation and die *in utero*. Here we report the generation of T-cell lineage-specific Brca2-deficient (*tBrca2*$^{-/-}$) mice using the *Cre-loxP* system. Mice with a flanked by loxP allele of *Brca2* were crossed to transgenic mice bearing *Cre* recombinase driven by the T cell-specific promoter *Lck*. Thymic cellularity and distribution of subset populations were normal in *tBrca2*$^{-/-}$ mutants. Thymocytes from *tBrca2*$^{-/-}$ mice underwent normal apoptosis in response to a variety of stimuli, and activated *tBrca2*$^{-/-}$ T cells had normal proliferative capacity. *tBrca2*$^{-/-}$ T cells were more likely than wild-type cells to undergo spontaneous apoptosis, but apoptosed normally in response to restimulation or DNA-damaging stress signals. Examination of metaphase spreads of *tBrca2*$^{-/-}$ T cells revealed that the chromosomes often exhibited aberrations such as breaks and tri-radial structures. The level of chromosomal abnormalities was enhanced in T cells from *tBrca2*$^{-/-}$*; p53*$^{-/-}$ double-mutant mice. However, *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells did not show the enhanced level of spontaneous apoptosis demonstrated by *tBrca2*$^{-/-}$ T cells, a difference that likely accounts for an increase in cell number and ^{3}[H]thymidine incorporation of double-mutant T cells in culture compared with either single mutant. Despite this increased T-cell number, the onset of T-cell lymphomas was only marginally accelerated in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice compared with *p53*$^{-/-}$ mice. Our results support a role for Brca2 in repairing spontaneous DNA lesions, and suggest that loss of Brca2 enhances the susceptibility of mouse T-lineage cells to chromosomal aberrations and deregulation of apoptosis in the absence of p53.**

INTRODUCTION

Approximately 5–10% of all female breast cancers in Western societies are hereditary (1, 2), and two disease-related genes have been identified: *BRCA1* and *BRCA2*. Mutations of *BRCA1* are found in 81% of families with a history of breast and ovarian cancers and in 52% of all hereditary breast cancers. Mutations of *BRCA2* are responsible for 32% of hereditary breast cancers in women (3). Both *BRCA1* and *2* encode large protein products of 1863 and 3418 amino acids, respectively. BRCA1 and BRCA2 do not share any amino acid homology, although these proteins have almost identical expression patterns both in mouse embryos (4) and in adult mouse tissues such as the thymus, testis, mammary gland, and ovary (5, 6). Both genes are essential for embryonic proliferation (7–13), but whereas Brca1-null mutants can be partially rescued by crossing into a p53- or p21-deficient genetic background, Brca2-null mutants are partially rescued only by crossing into a p53-deficient background (11, 13, 14).

Intensive research has identified many proteins capable of binding to either Brca1 or Brca2 (reviewed in Refs. 15, 16). Functional analyses suggest that the two gene products may have dual roles in maintaining genomic stability and regulating transcription. The first clues that the BRCA proteins might be involved in DNA damage response pathways were the discoveries that BRCA1 associates with the hRad50-hMre11-p95 complex (17) and Rad51 (18), and that BRCA2 binds to Rad51 (7, 19, 20). *Rad50* and *Rad51*, which belong to the *Rad52* epistasis group in *Saccharomyces cerevisiae*, are essential for DNA DSB[3] repair and homologous recombination (reviewed in Refs. 21, 22). Binding to Rad52 family members thus implies that the BRCA proteins are involved in pathways that repair DNA DSB. It has been shown that BRCA2 directly affects the DNA binding ability and intracellular localization of RAD51 (23), and that both BRCA1 and 2 are involved in homology-directed repair of chromosomal breaks (24, 25). Moreover, BRCA1 and 2 participate in transcription-coupled repair (26, 27), again establishing the roles of these proteins in DNA damage repair and clarifying their connections to carcinogenesis.

The involvement of BRCA2 in monitoring genome integrity has been supported by various other experiments involving complete or partial loss of Brca2 function in mice. In one study, homozygosity for a null mutation of *Brca2* caused both whole embryos and cells to be hypersensitive to IR (7). In another study, MEFs derived from animals homozygous for a truncation mutation of Brca2 proliferated poorly in culture and demonstrated defective repair of DNA DSB after exposure to ionizing radiation (28–30). These cells also displayed chromosomal abnormalities such as breaks, aberrant chromatid exchanges, centrosome amplifications, and translocations consistent with increased genomic instability (28, 31, 32). Interestingly, a small proportion of mutant mice with the Brca2 truncation survived to birth but then succumbed to thymic lymphomas by 3–4 months of age (28–30). These mutant mice also exhibit phenotypes similar to patients with Fanconi anemia, which led to a recent study revealing BRCA2 mutations in Fanconi anemia patients cell lines (33). Another mutant mouse strain with a homozygous deletion of exon 27 of Brca2 demonstrated an overall increased incidence of tumorigenesis and a shortened life span, additionally supporting a role for Brca2 as a tumor suppressor (34).

We and others have shown that Brca2-null mice die *in utero*, precluding the study of the effect of a *Brca2* null mutation in adult animals (7, 11, 13). Therefore, animals bearing conditional mutations

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[3] The abbreviations used are: DSB, double-strand break; MEF, mouse embryonic fibroblast; WT, wild-type; floxed, flanked by loxP; ES, embryonic stem; DN, double-negative; DP, double-positive; SP, single-positive; AICD, activation-induced cell death; mAb, monoclonal antibody; IL, interleukin; PI, propidium iodide; MMS, methyl methane sulfonate; RT-PCR, reverse transcription-PCR; TCR, T-cell receptor; NHEJ, nonhomologous end-joining; IR, γ-irradiation; FasL, Fas ligand; HDR, homology-directed recombination; SSA, single-strand annealing.

of Brca2 have been generated and their phenotypes characterized. For example, mice with mammary gland-specific loss of Brca2 develop mammary adenocarcinomas at high frequency after a long latency (35). An epithelia-specific Brca2 mutant develops mammary and skin tumors in the absence of p53, suggesting a synergy between Brca2 and p53 in tumor suppression of breast cancer (36). In the present study, we generated T lineage-specific Brca2 mutant mice using the *Cre-loxP* system (37). As *BRCA2* is highly expressed in the thymus in both humans and mice, the T-cell lineage is an excellent system in which to study the involvement of BRCA2 in DNA recombination, proliferation, and apoptosis. Mice in which *Brca2* was mutated only in the T-cell lineage ($tBrca2^{-/-}$ mice) were created by placing Cre recombinase under the regulation of the T cell-specific promoter *Lck* (38). Although the apoptotic response to DNA damage appeared normal in Brca2-deficient thymocytes, an increased incidence of chromosomal aberrations was observed in activated splenic $tBrca2^{-/-}$ T cells. Furthermore, these cells had a greater propensity than WT cells to undergo spontaneous apoptosis. This enhanced apoptosis was no longer observed when both Brca2 and p53 were mutated, but chromosomal abnormalities associated with loss of Brca2 were additionally increased in frequency in $tBrca2^{-/-}; p53^{-/-}$ T cells. These factors may have led to a general elevation in T-cell number in the double mutants, which may account for the increased ^{3}[H]thymidine incorporation exhibited by $tBrca2^{-/-}; p53^{-/-}$ T cells compared with T cells from either single mutant. However, the rate of onset of T-cell lymphomas was only slightly increased in $tBrca2^{-/-}; p53^{-/-}$ mice compared with $p53^{-/-}$ mice. Our findings suggest that Brca2 maintains genome integrity in stimulated mouse T lymphocytes, and that the simultaneous loss of both Brca2 and p53 synergistically drives genomic instability and deregulation of apoptosis.

MATERIALS AND METHODS

Generation of T Lineage-specific Brca2 Conditional Mutant Mice. A floxed allele of the *Brca2* gene was created by standard homologous recombination techniques in mouse ES cells. The targeting construct contained three loxP elements such that a hygromycin resistance cassette and *Brca2* exons 9 and 10 were flanked by loxP sites (Fig. 1*A*). E14K ES cells were electroporated with the targeting construct and cultured in the presence of 150 μg/ml hygromycin B (Sigma). Homologous recombinants ($Brca2^{hyg\text{-}fl9-10}$) were identified by PCR (using the primer pair 5′ CAGCCTCTGTTCCACATACACTTCATT 3′ and 5′ GCTAAATTTAATTGTTTTACAGCC 3′), and by Southern analysis. The hygromycin resistance cassette was removed from two targeted $Brca2^{hyg\text{-}fl9-10/+}$ ES clones by transient transfection with a cytomegalovirus promoter-driven construct encoding Cre recombinase, generating $Brca2^{fl9-10/+}$ ES clones. These $Brca2^{fl9-10/+}$ ES clones were used to derive two independent lines of $Brca2^{fl9-10/+}$ mice.

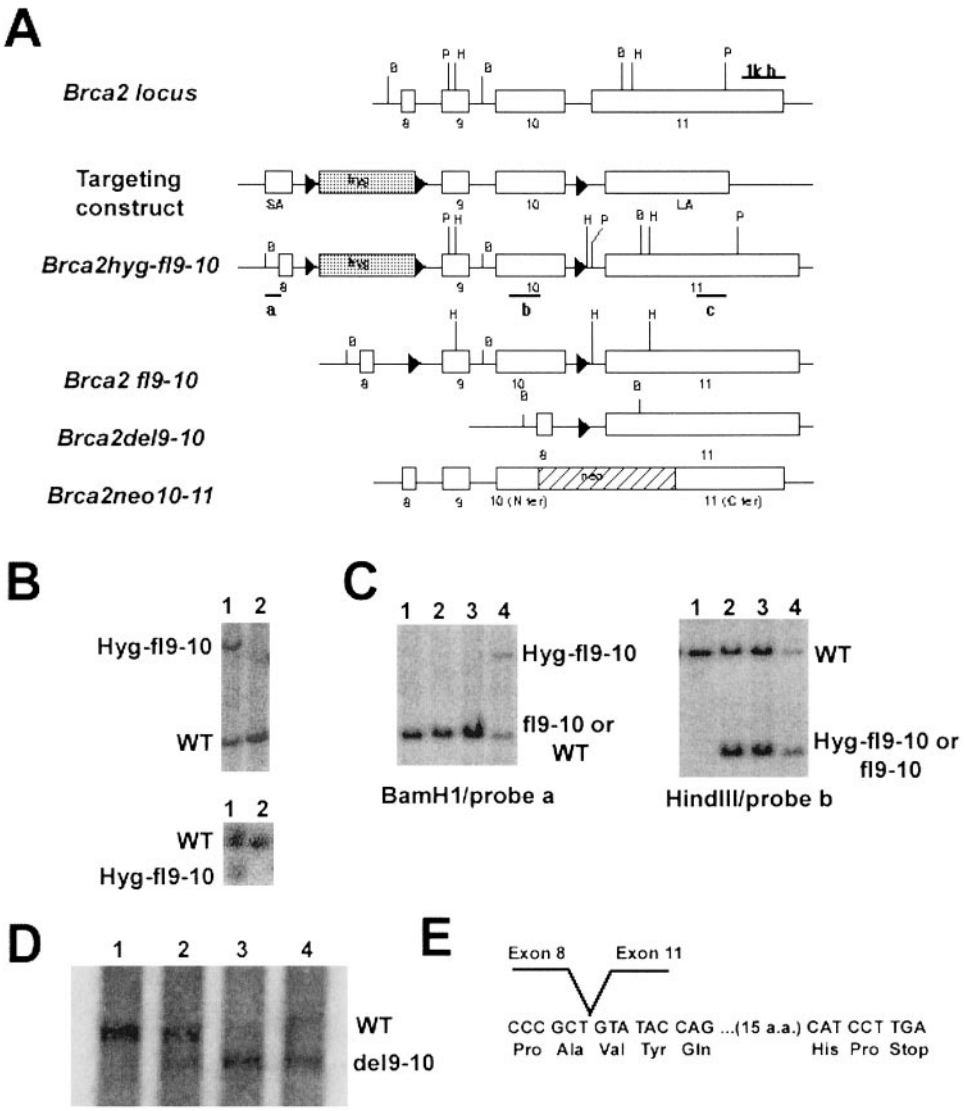

Fig. 1. Generation of the T-cell specific *Brca2* conditional mutation. *A*, schematic drawings of the partial WT *Brca2* locus, the targeting construct, and various mutated *Brca2* alleles, including the null $Brca2^{neo10-11}$ allele (13). Exons are indicated as *boxes* with *subscripted numbers*. LoxP sites are shown as *black triangles*. *B*, BamH1 site. *H*, HindIII site. *P*, Pst1 site. *SA*, short arm. *LA*, long arm. *hyg*, hygromycin resistance cassette. *neo*, neomycin resistance cassette. *B*, Southern blot analysis of genomic DNA derived from an ES clone with the rearranged $Brca2^{hyg\text{-}fl9-10}$ allele. *Top panel*, BamH1-digested genomic DNA hybridized with probe "a" in *A*. *Bottom panel*, Pst1-digested genomic DNA hybridized with probe "c" in *A*. *Lane 1*, $Brca2^{hyg\text{-}fl9-10/+}$. *Lane 2*, WT. *C*, Southern blot analysis of BamH1-digested (*left panel*) and HindIII-digested (*right panel*) genomic DNA from $Brca2^{hyg\text{-}fl9-10/+}$ ES cells transiently transfected with a cytomegalovirus-driven construct encoding Cre recombinase. Probes used were "a" in *A* for the *left panel* and "b" in *A* for the *right panel*. Clones that do not have the diagnostic restriction fragment for the Hy-fl9–10 allele in the BamH1 digest but contain the restriction fragment for the Hy-fl9–10 or fl9–10 allele are $Brca2^{fl9-10/+}$ cells. *Lane 1*, WT. *Lanes 2* and *3*, $Brca2^{fl9-10/+}$. *Lane 4*, $Brca2^{hyg\text{-}fl9-10/+}$. *D*, Northern blot of thymocyte RNA. *Lane 1*, WT. *Lane 2*, $Brca2^{fl9-10/+}$; *Lck-Cre*. *Lanes 3* and *4*, $Brca2^{fl9-10/fl9-10}$; *Lck-Cre*. Probe used was a 1.3 kb fragment from exon 11 of *Brca2*. *E*, sequence at the recombination site in the $Brca2\Delta^{9-10}$ allele. cDNA was generated using RNA from $tBrca2^{-/-}$ thymocytes, and PCR was performed using a primer pair binding to exons 7 and 11. Sequencing of the cloned PCR product revealed a frameshift linking exon 8 to exon 11 and a new stop codon ~60 nucleotides downstream of the recombination site.

To inactivate *Brca2* in T cells, the *Brca2*$^{fl9-10/+}$ mice were crossed to *Lck-Cre* transgenic mice expressing Cre recombinase under the control of the *Lck* proximal promoter (38, 39) to generate *Brca2*$^{fl9-10/+}$*; Lck-Cre* mice. These animals were intercrossed to produce *Brca2*$^{fl9-10/fl9-10}$*; Lck-Cre* mice in which Brca2 was inactivated in T lineage cells. The *Brca2*$^{fl9-10/+}$*; Lck-Cre* mice were also crossed to classically targeted *Brca2*$^{neo10-11/+}$ mice (13) to generate animals that carried one null *Brca2* allele and one floxed *Brca2* allele (*Brca2*$^{flox9-10/neo10-11}$*; Lck-Cre* mice). No differences in phenotype were observed between *Brca2*$^{fl9-10/fl9-10}$; *Lck-Cre* and *Brca2*$^{fl9-10/neo10-11}$; *Lck-Cre* animals, so that both were subsequently designated as *tBrca2*$^{-/-}$ mice. The *tBrca2*$^{-/-}$ phenotype was analyzed in both 129/C57BL/6 and C57BL/6 genetic backgrounds; no significant differences were observed. *Brca2*$^{fl9-10/fl9-10}$*; Lck-Cre* mice and *Brca2*$^{fl9-10/neo10-11}$*; Lck-Cre* mice were also crossed to *p53*$^{-/-}$ mice (Taconic) to create double-mutant *tBrca2*$^{-/-}$*; p53*$^{-/-}$ animals. All of the mice were analyzed at 6–10 weeks of age. At the end of each experiment, genomic DNA prepared from thymocytes of each mouse was subjected to Southern blotting to confirm the genotype, and to measure the level of deletion of exons 9 and 10 of the *Brca2*$^{fl9-10}$ allele (see below). All of the experiments were performed in compliance with the guidelines of the Ontario Cancer Institute Animal Care Committee.

PCR and Southern Blotting Analysis of Floxed Allele Recombination. Thymocytes from *tBrca2*$^{+/-}$ mice at 6 weeks of age were separated into populations of CD4- and CD8-negative (DN), and CD4- and/or CD8-positive (CD4$^+$ CD8$^+$, DP; CD4$^+$ CD8$^-$, CD4$^+$ SP; CD4$^-$ CD8$^+$, CD8$^+$ SP) cells using the MACS magnetic cell sorting system (Militenyi Biotech, Auburn, CA) according to manufacturer's protocols. For purification of T cells, lymph node cells were first purified using Lympholyte-M (Cedarlane) followed by depletion of B cells using Dynabeads biomagnetic cell separation system (DYNAL Biotech, Oslo, Norway), according to manufacturer's protocols. For PCR amplification of *Brca2*Δ^{9-10} allele, primers 5′ TCATTAGCTACACCACCAACC 3′ and 5′ TCACATGTTCTTTCTGGCAAGCATAAC 3′ were used. For PCR amplification of the *Brca2*$^{fl9-10}$ and WT allele, primers 5′ TTCTTGCTGGTTTTTGTTTTC 3′ and 5′ GCTAAATTTAATTGTTTTACAGCC 3′ were used. These primers bind on exon 8 and exon 9 of *Brca2* such that the PCR product of *Brca2*$^{fl9-10}$ allele is distinguished from WT with 50 bp longer in size (presence of a loxP site in *Brca2*$^{fl9-10}$). For Southern blotting, genomic DNA was digested with BamH1 and the blot hybridized to a probe derived from *Brca2* exon 8.

Flow Cytometry. Thymocytes, splenocytes, and lymph node cells were stained with the following mAbs (all from PharMingen): anti-CD4, anti-CD8, anti-Thy1.2, anti-TCR$\alpha\beta$, anti-B220, anti-IgM, anti-IgD, anti-CD44, and anti-CD25. Flow cytometric analyses were performed using a FACScalibur, and cells were sorted using a FACSVantage flow cytometer (Becton Dickinson).

AICD, Apoptosis, Proliferation, and Cell Cycle Assays. For AICD, total splenocytes were plated in RPMI 1640 (Life Technologies, Inc.) plus 10% FCS in a 10-cm dish containing plate-bound anti-CD3ϵ antibody at 5 μg/ml and soluble anti-CD28 antibody (PharMingen) at 100 ng/ml. After 24 h, cells were removed from activating signals and cultured in the presence of 50 units/ml IL-2 (Biosource International, Camarillo, CA) for 5 more days. On day 6, 5×10^5 viable cells were plated in 24-well plates in medium containing IL-2 plus various treatments. Cells were either left untreated (to determine spontaneous apoptosis), treated with 1 μg/ml anti-CD3 or 5 μg/ml FasL (to induce AICD restimulation), or treated with 1 Gy IR (Gammacell 40) or 0.1 μg/ml Adriamycin (Sigma; to induce DNA damage). After 24 h of treatment, apoptosis was determined by flow cytometry after staining with PI (Sigma). Exclusion of the stain was taken as evidence of survival. To assess the proliferation of activated T cells, 1×10^5 viable splenocytes were cultured in a 96-well plate and pulsed with ^{3}[H]thymidine for 16 h. For thymocyte apoptosis, 1×10^6 cells were either left untreated, or treated with UV (20 or 40 mJ; UV crosslinker; Stratagene), ionizing IR (0.5, 1, 2, or 3 Gy; Gammacell 40), or the alkylating agent MMS (10, 20, 50, 100 nM; Sigma). Treated thymocytes were then cultured in a six-well plate (Falcon) in RPMI 1640 plus 10% FCS for 24 h. Apoptosis was determined by flow cytometry after staining harvested cells with Annexin V-FITC and PI (Apoptosis Detection kit; R&D Systems). For cell cycle analysis, 1×10^6 thymocytes either left untreated or treated as above were cultured for 24 h and fixed in 70% ethanol. Activated T cells (1×10^6) were treated with 1 Gy of IR and cultured for 6 or 24 h before being fixed in 70% ethanol. Fixed cells were then subjected to RNase (Sigma) and stained with PI for 30 min before analysis by flow cytometry.

Northern Blotting and RT-PCR Analysis. Total RNA was extracted from thymocytes using TRIzol (Life Technologies, Inc.) according to the manufacturer's protocol, and 30 μg were used for Northern blotting. A 1.3 kb EcoRI/EcoRV fragment from exon 11 of mouse *Brca2* corresponding to amino acids 677-1109 was used as the probe. cDNA was generated using the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol. RT-PCR was performed using the primer pair 5′ TCATTAGCTACACCACCAACC 3′ and 5′ TCACATGTTCTTTCTGGCAAGCATAAC 3′. PCR products were cloned using the TOPO TA Cloning kit (Invitrogen) and sequenced.

Western Blotting. Thymocytes (5×10^6) were either left untreated or γ-irradiated (10 Gy) and cultured for 6 h in RPMI 1640 plus 10% FCS followed by lysis and preparation of protein extracts using standard protocols. Antibodies reactive to murine p53 (CM5; Novocastra) and β-actin (Sigma) were used for immunoblotting.

Cytogenetic Analyses. Lymph node cells (5×10^6) purified using Lympholyte-M (Cedarlane) were stimulated with plate-bound anti-CD3ϵ (5 μg/ml) and soluble anti-CD28 (1 μg/ml) antibodies for 24 h to induce proliferation. Cells were then cultured in 50 units/ml IL-2 for 24 h before treatment with 100 ng/ml Colcemid (Boehringer Mannheim) for 2 h to induce arrest in the mitotic state. The condensed metaphase chromosomes were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma) and viewed with an Zeiss Axioplan 2 Imaging microscope equipped with a CCD camera. The images were acquired using ISIS software (Metasystems, Stuttgart, Germany) and processed using Adobe Photoshop software.

Histological Analyses. Mice in distress or exhibiting obvious tumors were sacrificed and subjected to histopathological evaluation. Tissues and tumors were fixed in 10% buffered formalin followed by dehydration and embedding in paraffin. Paraffin blocks were sectioned at 3 μm and stained with H&E. Identification of B and T cells was performed by dewaxing 3 μm sections in toluene and rehydrating through graded alcohol solutions to water. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide. After pretreatment with pepsin digestion, slides were incubated in primary antibody for 1–2 h at room temperature. After washing in PBS, secondary incubations were carried out with biotin-conjugated antirabbit CD3 (DAKO; used at 1:150) or antirat B220 (PharMingen; 1:400) IgG antibodies followed by streptavidin-horseradish peroxidase for 30 min. Immunoreactivities were revealed by incubation in 3-amino-9-ethylcarbazol. Slides were counterstained in hematoxylin and mounted with Crystal Mount.

Statistical Analysis. Survival curves of mice were plotted using SigmaPlot 5.0 and analyzed with Prism software, which uses a log-rank test to determine statistically significant differences between tumor-free survival curves of different mice.

RESULTS

Generation of T Cell Lineage-specific Brca2-deficient Mice. The *Cre-loxP* system (37) was used to generate mice with a conditionally targeted *Brca2* allele in T cells. The targeting construct, which was designed to delete *Brca2* exons 9 and 10 via *Cre*-mediated recombination (Fig. 1*A*), was electroporated into ES cells. Successfully targeted clones (*Brca2*$^{hyg-fl9-10}$) were identified by Southern blotting (Fig. 1*B*). *Brca2*$^{hyg-fl9-10}$ ES clones were then transiently transfected with Cre recombinase. Transfected ES clones in which the hygromycin resistance cassette was removed but the floxed exons 9 and 10 were retained (*Brca2*$^{fl9-10}$) were identified by Southern blotting (Fig. 1*C*). Two independent *Brca2*$^{fl9-10}$ ES clones were injected into blastocysts, and the mutation was successfully transmitted to the germ line. Mice heterozygous or homozygous for *Brca2*$^{fl9-10}$ were viable and fertile, as offspring were obtained according to the expected Mendelian frequencies. To inactivate *Brca2* in T cells, *Brca2*$^{fl9-10}$ mice were crossed to *Lck-Cre* transgenic animals as described in "Materials and Methods" to yield *tBrca2*$^{-/-}$ mice bearing the *Brca2*Δ^{9-10} allele in T lymphocytes.

To confirm the deletion of the floxed exons in the *Brca2*$^{fl9-10}$ allele, Northern blotting was performed in which a probe specific for *Brca2* exon 11 was hybridized to total RNA prepared from WT and

mutant thymocytes. A transcript of ~11 kb was detected in RNA from WT and *tBrca2*$^{+/-}$ thymocytes, whereas a shorter transcript of ~10 kb was detected in RNA from *tBrca2*$^{+/-}$ and *tBrca2*$^{-/-}$ thymocytes (Fig. 1*D*). This result suggests that Cre recombinase was efficiently expressed and successfully excised exons 9 and 10 of the floxed *Brca2* allele. No other transcripts were identified using this probe, indicating that splice variants of *Brca2* exons 9 and 10 do not exist, in agreement with a previous report (6). To additionally confirm the deletion of exons 9 and 10, cDNA obtained from WT or *tBrca2*$^{-/-}$ thymocytes was subjected to PCR amplification using primers located on either side of a region including the deletion and the site of the expected stop codon (5′ primer on exon 7 and 3′ primer on exon 11). WT cDNA analyzed in this assay generated a PCR product of 1137 bp, whereas a PCR product of 300 bp was produced when *tBrca2*$^{-/-}$ cDNA was used as the template (data not shown). Nucleotide sequencing revealed that a frameshift had indeed occurred in the mutant cells and that a stop codon had been introduced 20 amino acids downstream of the recombination site (Fig. 1*E*). Thus, even if the *Brca2*Δ^{9-10} transcript were to be translated, it would produce only the first 219 residues of Brca2, a small fraction of the WT protein of 3328 residues (6).

Brca2 Is Not Required for T-Lineage Development. Thymocyte development is divided into several stages, which can be distinguished after the expression of the TCR (composed of α and β chains), and the cell surface markers CD4 and CD8 (Fig. 2*A*). The most immature thymocytes appear as CD4$^-$ CD8$^-$ (DN) cells, which constitute ~5% of the total thymocyte population in an adult thymus. This is followed by maturing thymocytes coexpressing CD4 and CD8 (DP). This population constitutes the majority of thymocytes in the thymus, although most of them are destined to die by negative selection. The most mature thymocytes lose expression of either CD4 or CD8, becoming either CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ (SP) thymocytes. Both subsets of SP thymocytes then migrate to peripheral lymphoid organs such as the spleen and lymph nodes and take up residence as naïve

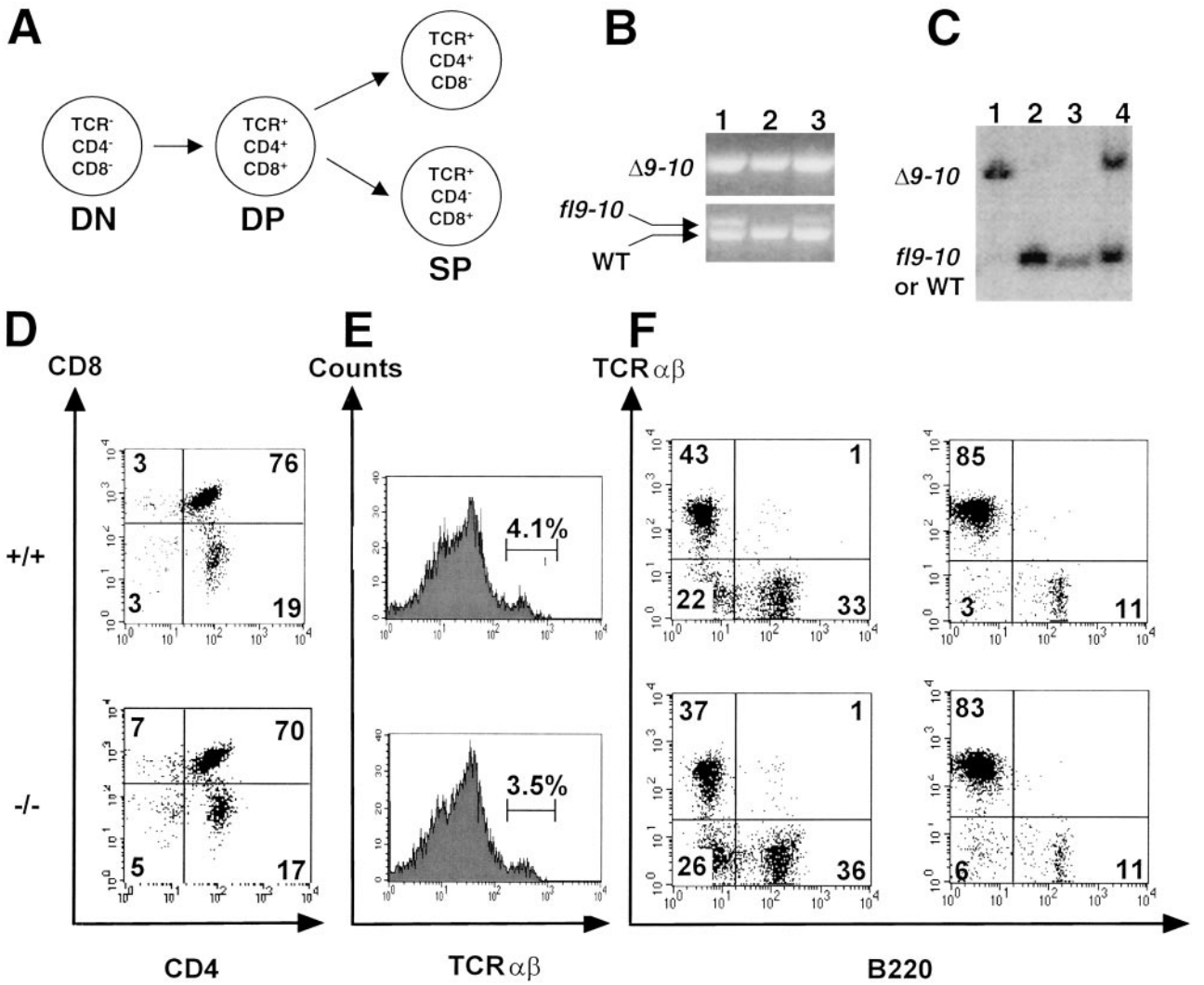

Fig. 2. Recombination of *Brca2*$^{fl9-10}$ allele by Cre recombinase, normal development of lymphoid tissue in *tBrca2*$^{-/-}$ mice, and normal expression of TCR-α and -β chains. *A*, stages of thymocyte development as distinguished by the expression of the TCR and the cell surface markers CD4 and CD8. *B*, amplification of a PCR product indicating the recombination of the *Brca2*$^{fl9-10}$ allele (*top panel*), and amplification of PCR products of the *Brca2*$^{fl9-10}$ allele and WT allele (*bottom panel*), using template DNA from thymocytes or T cells at different developmental stages from a *tBrca2*$^{+/-}$ mouse. *Lane 1*, DN thymocytes. *Lane 2*, DP or SP thymocytes. *Lane 3*, mature T cells from spleen and lymph nodes. *C*, recombination at the *Brca2*$^{fl9-10}$ allele in thymocytes of different genotypes. Southern blot analysis of BamH1-digested genomic DNA hybridized to probe "a" in Fig. 1*A*. *Lane 1*, *Brca2*$^{fl9-10/fl9-10}$; *Lck-Cre*. *Lane 2*, *Brca2*$^{fl9-10/fl9-10}$. *Lane 3*, *Brca2*$^{fl9-10/+}$. *Lane 4*, *Brca2*$^{fl9-10/+}$; *Lck-Cre*. Cre recombinase expressed by the transgene efficiently excises exons 9 and 10 from the *Brca2*$^{fl9-10}$ allele. *D–F*, representative flow cytometric analyses of cells obtained from WT (*top*) and *tBrca2*$^{-/-}$ (*bottom*) mice. *D*, CD4 and CD8 expression in total thymocytes. *Numbers within quadrants* indicate the relative percentages of positively stained cells. *E*, TCR-$\alpha\beta$ expression on gated CD4$^+$CD8$^+$ thymocytes. Percentages of TCR-$\alpha\beta^+$ cells are as indicated. *F*, percentages of mature T cells (expressing TCR-$\alpha\beta$) and B cells (expressing B220) in total splenocytes (*left*) or total lymph node cells (*right*). Experiments were repeated three times using 6 mice of each genotype.

mature T cells. Mature T cells are activated by the engagement and aggregation of their TCRs by specific antigen. *In vitro*, T-cell activation is commonly induced by adding anti-CD3ϵ and anti-CD28 antibodies. Both *in vivo* and *in vitro*, activation triggers the proliferation of mature T cells (40, 41).

The expression of the T cell-specific *Lck* proximal promoter has been well characterized in mouse models in which *Lck-Cre* transgenic mice have been used to generate T lymphocyte-specific gene knockouts (38, 39). It has been shown that the expression of the *Lck-cre* gene commences early in DN thymocytes. We have carried out PCR and Southern blotting analyses to determine the onset of *Lck*-induced Cre recombinase expression in thymocytes of our $tBrca^{+/-}$ mice. The presence of the deleted $Brca2\Delta^{9-10}$ allele is inferred from the appearance of a specific PCR product of 300 bp that results from PCR amplification using the RT-PCR primers described in "Materials and Methods." This assay detected the presence of the $Brca2\Delta^{9-10}$ allele in DN, DP, and SP thymocytes, as well as in peripheral T cells of $tBrca2^{-/-}$ mice (Fig. 2*B, top panel*), indicating that Cre-mediated deletion of the $Brca2^{fl9-10}$ allele had occurred in these cells. Activation of *Lck-cre* expression at these stages is consistent with a previous report (39).

To determine the extent of Cre-mediated recombination, DNA obtained from $tBrca2^{+/-}$ thymocytes was used as the template for a set of primers that amplifies both the $Brca2^{fl9-10}$ and the WT *Brca2* but not the $Brca2^{\Delta 9-10}$ allele (by binding to sequences on exon 8 and 9) and results in products differing in size by 50 bp (with or without a loxP site between exon 8 and 9). We reasoned that the greater the amount of the longer PCR product (top band) detected, the greater the number of T cells present with a nonrecombined $Brca2^{fl9-10}$ allele. As shown in *Lane 1* of Fig. 2*B* (*bottom panel*), in DN thymocytes, the intensity of the upper PCR product (representing the $Brca2^{fl9-10}$ allele) was decreased relative to the lower band (the WT allele). Thus, some DN cells had achieved recombination of the $Brca2^{fl9-10}$ allele but most cells remained unrecombined. However, in DP/SP thymocytes (Fig. 2*B, Lane 2*), the $Brca2^{fl9-10}$ allele was no longer amplified by PCR, suggesting that the $Brca2^{fl9-10}$ allele had recombined in all of the cells by this stage. Because all of the DP/SP thymocytes achieved recombination of the $Brca2^{fl9-10}$ allele, the peripheral T cells developing from them should also have the recombined $Brca2^{fl9-10}$ allele, as evident in Fig. 2*B, Lane 3*. The faint $Brca2^{fl9-10}$ PCR product visible in Fig. 2*B, Lane 3* is probably because of minor B-cell contamination.

Southern analyses of genomic DNA obtained from thymocytes (Fig. 2*C*), splenocytes, and lymph node cells (data not shown) of $tBrca2^{-/-}$ mice confirmed the deletion of *Brca2* exons 9 and 10 in these cell types. Moreover, almost all of the thymocytes, ~20% of splenocytes, and 80% of lymph node cells from $tBrca2^{-/-}$ mice exhibited the recombined $Brca2^{fl9-10}$ allele. These proportions correlate well with the percentages of T-lineage cells in WT thymus, spleen, and lymph nodes. Thus, our data confirm that *Brca2* deletion occurs early in immature thymocytes and assure us that any effects we observe on T-lineage cell development are most likely the result of the loss of Brca2 function.

The size and total cell numbers of the thymus, spleen, and lymph nodes (axillary and mesenteric) were approximately equal in WT, $tBrca2^{+/-}$, and $tBrca2^{-/-}$ animals (data not shown). Flow cytometric analyses of the expression of CD4, CD8, TCR-$\alpha\beta$, and B220 (Fig. 2, *D–F*), and Thy1.2, CD44, CD25, IgM, and IgD (data not shown), indicated that the various subpopulations of lymphoid cells were intact. Thus, T-lymphocyte development is normal in the absence of Brca2. V(D)J recombination and cell surface expression of the TCR-β chain occurs during the DP stage of thymocyte development, which is followed shortly by the recombination of α chain (42). These processes, which involves DNA DSB rejoining using NHEJ, was not defective in $tBrca2^{-/-}$ thymocytes (Fig. 2, *E* and *F*). This additionally supports its role in regulating DSB repair through homologous recombination and not NHEJ (25). In comparison, fetal liver B lymphocytes in mice with Brca2 truncation also exhibit normal V(D)J recombination at the *IgH* locus (28).

Brca2 Is Not Required for Recovery from DNA-damaging Stress in Mouse Thymocytes *in Vitro*. To evaluate whether Brca2 plays a role in recovery from DNA-damaging stress in lymphoid cells, $tBrca2^{-/-}$ and control thymocytes were treated with various DNA-damaging agents and stained 24 h later with Annexin V/PI to determine apoptosis. Specifically, thymocytes from WT, $tBrca2^{+/-}$, and $tBrca2^{-/-}$ mice were subjected to increasing doses of UV, IR, or MMS as shown in Fig. 3*A*. At the 24 h time point, $tBrca2^{-/-}$ thymocytes were just as able as control thymocytes to recover from damage and survive. Although it has been shown that lymphoid cells generally react to DNA-damaging stress by undergoing apoptosis instead of cell cycle arrest (43, 44), we monitored the cell cycle profile of viable thymocytes using PI staining to determine whether loss of Brca2 could possibly affect the induction of cell cycle arrest. Like $tBrca2^{+/-}$ thymocytes, most $tBrca2^{-/-}$ thymocytes remained resting in the G_0/G_1 phase of the cell cycle (Fig. 3*B*). Treatment of these cells with UV did not alter the percentages of G_0/G_1, S, and G_2/M phases of $tBrca2^{+/-}$ and $tBrca2^{-/-}$ thymocytes. Interestingly, both $tBrca2^{+/-}$ and $tBrca2^{-/-}$ thymocytes showed a similar increase in S phase cells after IR. This could be a manifestation of radiation-induced differentiation of thymocytes to preleukemic cells (45). Taken together, we can exclude the possibility that the absence of Brca2 affects the cell cycle of thymocytes treated with DNA-damaging stress.

We then subjected thymocytes from double mutant $tBrca2^{-/-}$; $p53^{-/-}$ and $tBrca2^{-/-}$; $p53^{+/-}$ mice to the same treatment regimens as in Fig. 3*A* and analyzed their apoptotic responses (Fig. 3*C*). In the absence of p53, thymocytes acquired substantial resistance to IR but no resistance to UV- or MMS-induced cell death. This pattern was observed whether or not Brca2 was present. In addition, stabilization of the p53 protein after IR was unaffected in $tBrca2^{-/-}$ thymocytes (Fig. 3*D*). These data indicate that, at least in murine thymocytes, Brca2 is not required for apoptosis induced by three very different DNA-damaging agents.

Normal Proliferation of Activated $tBrca2^{-/-}$ T Cells. The Brca2 null mutation has been associated with the up-regulation of the cell cycle regulators p53 and $p21^{WAF1/Cip1}$ in embryonic cells (11, 13). MEFs bearing the Brca2 truncation mutation also demonstrated a block in cell cycle entry (28). To test if an absence of Brca2 affected the proliferation of mature T cells, we assessed the proliferative capacity of $tBrca2^{-/-}$ splenic T cells. Splenocytes from control and $tBrca2^{-/-}$ mice were activated by culturing them with plate-bound anti-CD3ϵ antibody plus soluble anti-CD28 antibody for 24 h. The cells were then removed from the activating signals and cultured in the presence of recombinant IL-2. Although T-cell numbers in the spleen are low (about 20–30% of total splenocytes), $tBrca2^{-/-}$ T cells were able to proliferate normally so that by day 6, the culture was enriched with T cells bearing the recombined $Brca2^{fl9-10}$ allele (Fig. 4*A*). The normal growth capacity of $tBrca2^{-/-}$ T cells at 72 h after activation was additionally quantified by performing ^{3}[H]thymidine incorporation assays (Fig. 4*B*). T cells from $tBrca2^{-/-}$ mice expanded at a rate similar to that of $tBrca2^{+/-}$ and WT controls. Thus, activated mouse T cells show normal proliferative capacity in the absence of Brca2.

Enhanced Spontaneous Apoptosis in Proliferating $tBrca2^{-/-}$ T Cells. Although $tBrca2^{-/-}$ thymocytes were no more likely than WT cells to undergo apoptosis in response to DNA-damaging agents, we

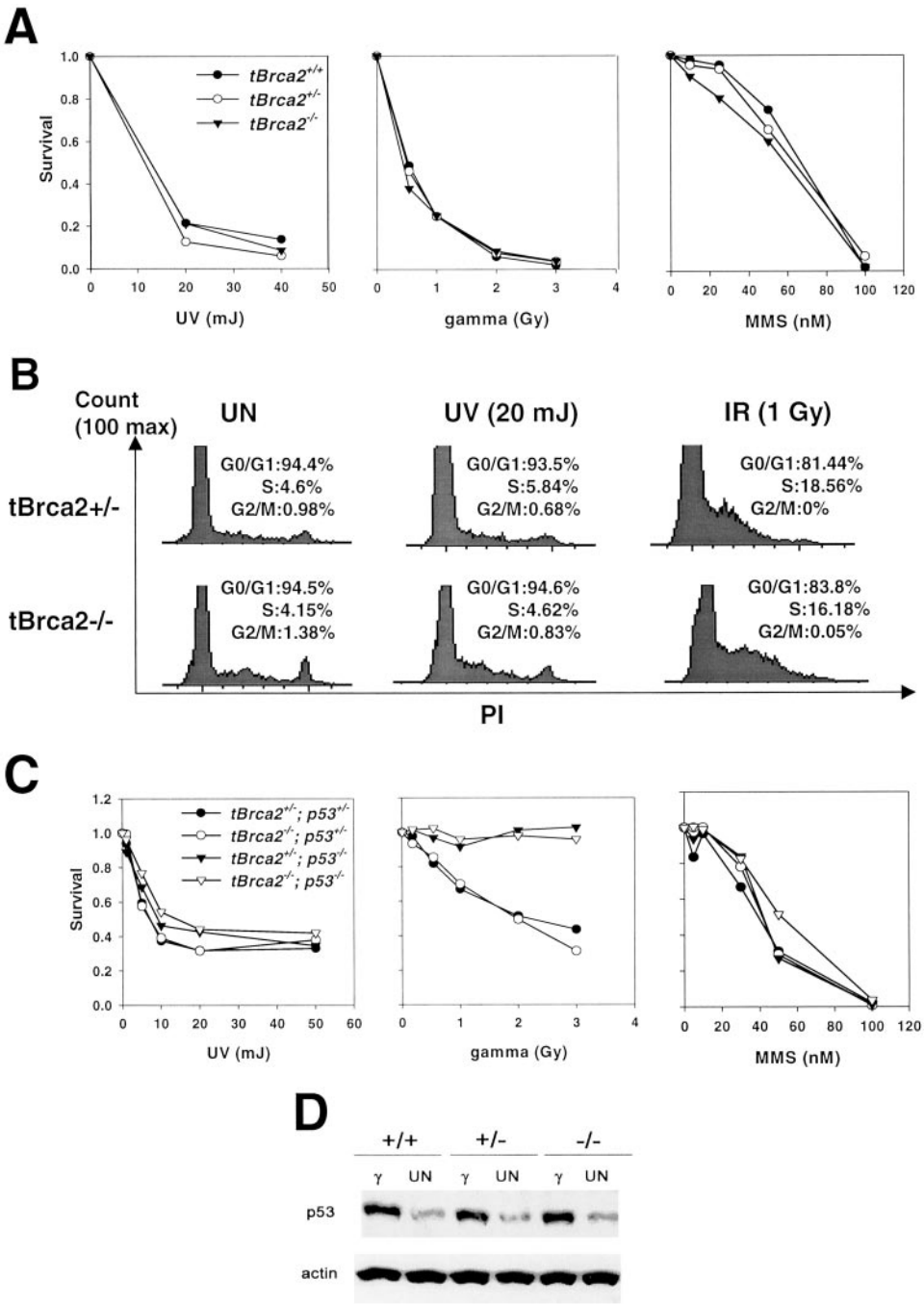

Fig. 3. Normal DNA damage-induced apoptosis of *tBrca2*$^{-/-}$ thymocytes. *A*, apoptotic response to DNA-damaging stress in the absence of Brca2. Thymocytes from *tBrca2*$^{+/+}$, *tBrca2*$^{+/-}$, and *tBrca2*$^{-/-}$ mice were subjected to increasing doses of UV, IR, or MMS as indicated. Cell survival was quantified at 24 h after treatment by flow cytometric analysis of cells stained with Annexin V-FITC/PI. Results are expressed as the fraction of cells excluding the stain in treated *versus* untreated populations. Data shown are representative of three independent experiments. *B*, cell cycle profiles of thymocytes after treatment with DNA-damaging stress. Thymocytes from *tBrca2*$^{+/-}$ and *tBrca2*$^{-/-}$ mice were left untreated (*UN*) or treated with the indicated doses of UV irradiation (*UV*) or IR. Cells were fixed and stained with PI at 24 h after treatment. Percentages of cells in G_0/G_1, S, and G_2/M phases were indicated. To show the lower G_2/M peak, the *Y* axis of each panel was cut off at count = 100. Data shown are representative of two independent experiments. *C*, apoptotic response to DNA-damaging stress in the absence of Brca2 and p53. Thymocytes from *tBrca2*$^{+/-}$ or *tBrca2*$^{-/-}$ mice with or without p53 were treated as indicated and their survival quantified as in *A*. A lack of Brca2 did not render *p53*$^{-/-}$ cells any more sensitive to DNA-damaging stress. Results are expressed as in *A* and are representative of three independent experiments. *D*, stabilization of p53 after IR. Thymocytes from *tBrca2*$^{+/+}$, *tBrca2*$^{+/-}$, and *tBrca2*$^{-/-}$ mice were either left untreated (*UN*) or subjected to IR (γ). Protein lysates of harvested cells were immunoblotted to detect p53. Each lane contains an equal amount of protein (20 μg).

investigated spontaneous and AICD-induced apoptosis in proliferating *tBrca2*$^{-/-}$ T cells. During AICD, activated T cells are readily killed after restimulation via signals such as the engagement of the TCR by anti-CD3ϵ and anti-CD28, or by binding of the Fas receptor to FasL (46). We first activated *tBrca2*$^{-/-}$ and control T cells with anti-CD3ϵ plus anti-CD28 antibodies and cultured them for 6 days in IL-2. Although we observed variations between individual experiments, we consistently observed that more *tBrca2*$^{-/-}$ T cells died spontaneously in culture compared with their respective controls in all four of the experiments conducted (Fig. 5*A*, experiments 1–4). This result suggested that activated T cells, which are constantly proliferating, are more prone to spontaneous apoptosis in the absence of Brca2. To study whether this effect was dependent on p53, T cells of *tBrca2*$^{+/-}$*; p53*$^{-/-}$ and *tBrca2*$^{-/-}$*; p53*$^{-/-}$ double-mutant mice were subjected to the same culture conditions. As shown in experiments 3 and 4 (Fig. 5*A*), activated *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells were much less likely to undergo spontaneous death than were *tBrca2*$^{-/-}$ T cells, indicating that signals mediated through p53 are important for the increased spontaneous death of activated *tBrca2*$^{-/-}$cells. A similar set of experiments was performed in which T cells activated as above were subjected to either restimulation using anti-CD3ϵ antibody or FasL (to induce AICD), or to the DNA-damaging agents IR or Adriamycin. In both cases, *tBrca2*$^{-/-}$ and control T cells demonstrated comparable folds of increase in apoptosis (ratio of apoptosis in treated *versus* untreated populations; Fig. 5*B*), although more *tBrca2*$^{-/-}$ T cells die spontaneously after activation. Similar to Fig. 3*B*, we examined the cell cycle profiles of activated *tBrca2*$^{+/-}$ and *tBrca2*$^{-/-}$ T cells after exposure to IR (Fig. 5*C*). Again the majority

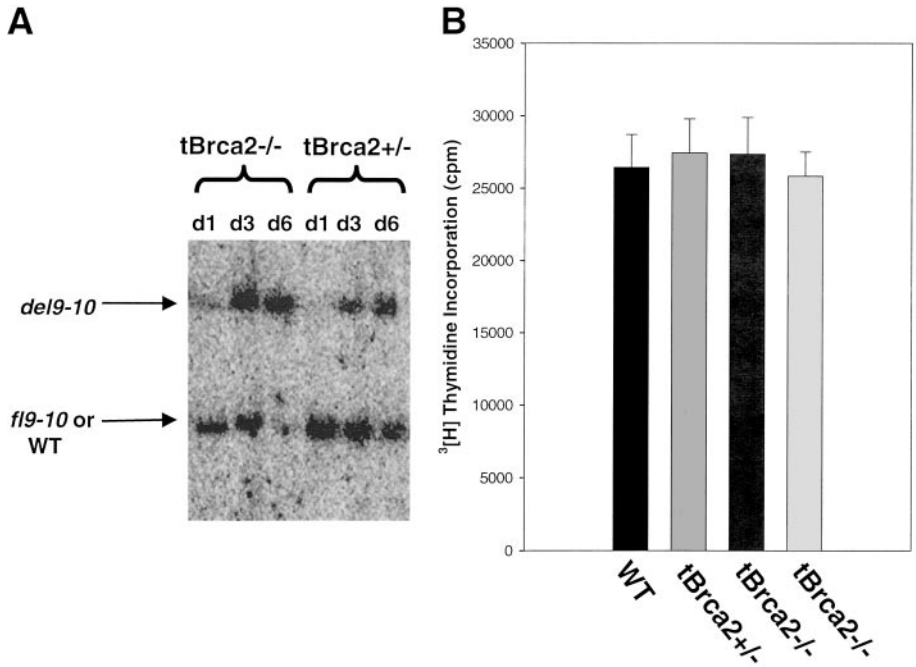

Fig. 4. Normal proliferation of activated *tBrca2*$^{-/-}$ T cells. *A*, enrichment for the recombined floxed Brca2 allele in cultures of proliferating *Brca2*$^{fl9-10/fl9-10}$*; Lck-Cre* T cells. Splenocytes from *Brca2*$^{fl9-10/fl9-10}$*; Lck-Cre* and *Brca2*$^{fl9-10/+}$*; Lck-Cre* mice were stimulated with anti-CD3ϵ, anti-CD28, and IL-2 as described in "Materials and Methods" and harvested on days 1, 3, and 6 after activation. Genomic DNA was isolated, digested with BamHI, and subjected to Southern blotting using the same probe as in Fig. 2*C*. *B*, proliferation of the T cells in *A* at 72 h after activation as quantified by ^{3}[H]thymidine incorporation. Results are representative of three independent experiments; *bars*, ±SD.

of the activated T cells are resting in G_0/G_1 phase. At 6 h after IR both mutant and control cells exhibited a similar decrease in G_0/G_1 populations and increase in G_2/M populations, which were restored to near normal levels 24 h later. These data suggest that the absence of Brca2 has no effect on proliferation blockage of activated T cells after treatment with IR. Similar resistance to apoptosis induced by AICD or DNA-damaging stress was also observed in activated *tBrca2*$^{-/-}$*; p53*$^{-/-}$ and *tBrca*$^{+/-}$*; p53*$^{-/-}$ T cells (Fig. 5*D*). Thus, a lack of Brca2 predisposes T cells only to death by spontaneous apoptosis, an event that involves p53 signaling.

Chromosomal Aberrations in *tBrca2*$^{-/-}$ and *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T Cells. An elevated level of spontaneous chromosomal aberrations had been reported previously in cells with the Brca2 truncation (28, 31, 32). Therefore, we examined chromosomal integrity in activated *tBrca2*$^{-/-}$ T cells and controls using cytogenetic analysis. Lymph node T cells were stimulated with plate-bound anti-CD3ϵ and soluble anti-CD28 antibodies, cultured in the presence of IL-2, and arrested by Colcemid treatment. Cytogenetic analysis of 4′,6-diamidino-2-phenylindole dihydrochloride-stained metaphase spreads revealed increased genomic instability in activated *tBrca2*$^{-/-}$ T cells compared with controls (Table 1). Of 256 metaphase spreads from *tBrca2*$^{-/-}$ T cells, 37 (14.5%) exhibited aneuploidy. Furthermore, compared with WT, *Lck-Cre*, or *tBrca2*$^{+/-}$ cells, there was a significant increase in the frequency of aberrations such as chromatid or chromosomal breaks, tri-radial structures, and chromosome fragments. The increased level of chromosomal abnormalities in *tBrca2*$^{-/-}$ T cells agrees with the earlier finding in MEFs bearing the Brca2 truncation of increased chromatid/chromosomal breaks and tri- and quadri-radial structures, anomalies thought to be because of defective homologous recombination (28). Moreover, the low number of chromosomal aberrations observed in *Lck-Cre* T cells indicates that the expression of Cre recombinase driven by the *Lck* promoter does not necessarily result in endogenous breaks, contrary to other reports (47, 48). Parallel cytogenetic analyses of T cells from *p53*$^{-/-}$ and *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice showed that loss of p53 in *tBrca2*$^{-/-}$ T cells significantly increased aneuploidy and the frequency of other cytogenetic aberrations (chromatid breaks and tri-radials). Specifically, the mean number of aberrations per cell rose from 0.19 in *tBrca2*$^{-/-}$ cells to 1.04 in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ cells. These observations are in clear agreement with previous studies suggesting that Brca2 is involved in monitoring genome integrity (28, 31, 32). Moreover, our chromosomal data taken together with the proliferation and apoptosis data shown in Figs. 4 and 5 support the hypothesis that chromosomal abnormalities are able to accumulate in the absence of p53 because signaling to induce cell cycle arrest or apoptosis is abrogated.

Increased Cell Number of *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T Cell. To investigate whether the chromosomal aberrations in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells were linked to cell cycle deregulation, *tBrca2*$^{+/-}$*; p53*$^{-/-}$ and *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells were activated with anti-CD3ϵ and anti-CD28 antibodies followed by culture in IL-2. After 24 and 72 h, the capacity of these cells to proliferate was measured by ^{3}[H]thymidine incorporation. At 24 h, cells of all genotypes showed similar proliferative capacities (Fig. 6). However, at 72 h, in contrast to activated *tBrca2*$^{-/-}$ cells, the level of ^{3}[H]thymidine uptake of activated *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells was greatly increased over that of *tBrca2*$^{+/-}$*; p53*$^{-/-}$ controls, which in turn was higher than activated T cells from either *tBrca2*$^{+/-}$ or *tBrca2*$^{-/-}$ mice. Whereas this increase could represent a bona fide increase in proliferative capacity, we feel it more likely reflects the comparatively reduced rate of apoptosis of double mutant cells. Thus, at this juncture, it appears that loss of Brca2 in T cells deregulates apoptotic signaling mediated by p53.

Incidence and Rate of Onset of T-Cell Lymphomas in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ Mice. Surviving mice bearing a mutation eliminating the COOH terminus of Brca2 all eventually died of thymic lymphomas at 3–4 months of age (29, 30). However, *tBrca2*$^{-/-}$ mice ($n = 19$) have not developed any kinds of tumors and have survived for at least 500 days. Clinical studies have shown that p53 is more frequently inactivated in human tumors bearing mutated *BRCA1* or *BRCA2* alleles than in sporadic breast or ovarian tumors (49, 50). Lymphomas in Brca2 truncation mutant mice also exhibit dysfunction of p53 or other spindle checkpoint proteins (51). These results, together with our demonstration that Brca2 and p53 deficiencies act synergistically in driving genomic instability and abnormal apoptosis, prompted us to monitor spontaneous tumor development in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice.

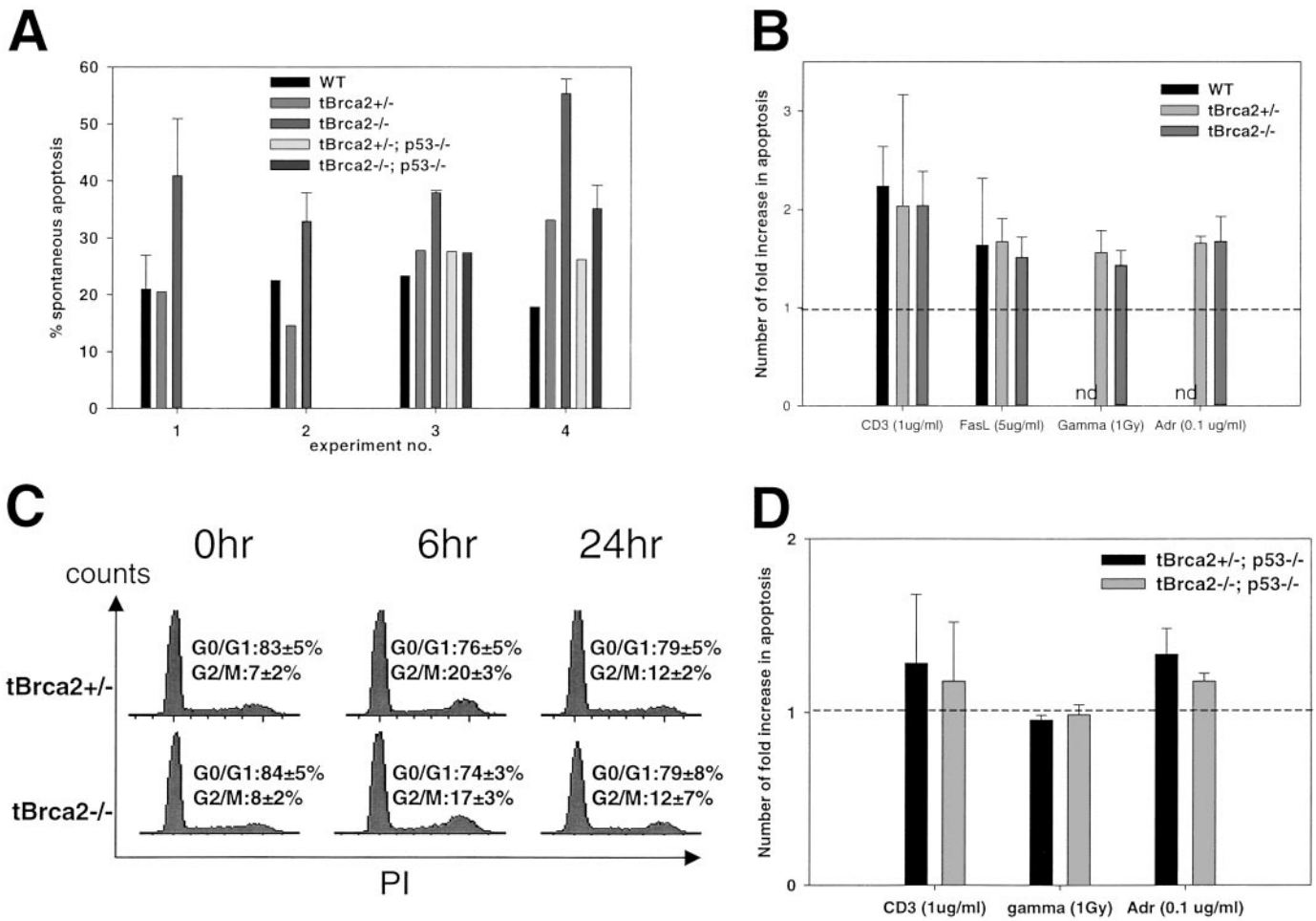

Fig. 5. Increased spontaneous apoptosis but normal sensitivity to restimulation or DNA-damaging stress in activated *tBrca2*$^{-/-}$ T cells. *A*, effect of Brca2 deficiency on spontaneous apoptosis of proliferating T cells. Splenocytes were stimulated and cultured for 6 days with anti-CD3ϵ, anti-CD28, and IL-2 as described in "Materials and Methods." Apoptosis was measured by PI exclusion and flow cytometric analysis. The cumulative results of four independent experiments are shown. The mean percentage of apoptotic cells is shown where more than one animal of a given genotype was analyzed. *B*, effect of Brca2 deficiency on AICD of T cells. T cells of the indicated genotypes were treated with the indicated stimuli after activation, and the level of induced apoptosis was quantified as in *A*. Results shown are the mean fold increase in apoptosis over uninduced controls of the mean of three trials in which each value was derived from three samples. *nd*, not determined. *C*, cell cycle profiles of activated T cells after treatment with IR. T cells from *tBrca2*$^{+/-}$ and *tBrca2*$^{-/-}$ mice were exposed to 1 Gy of IR and examined 6 and 24 h after treatment. Percentages of cells in G_0/G_1 and G_2/M phases were indicated. Results shown are the mean percentage of the mean of four samples. *D*, effect of combined Brca2 and p53 deficiency on AICD of T cells. T cells of the indicated genotypes were treated with the indicated stimuli after activation and the level of apoptosis was quantified as in *A*. Results shown are the mean fold increase in apoptosis over uninduced controls of the mean of three trials; *bars*, ±SD.

Previous studies have established that *p53*$^{-/-}$ mice are highly susceptible to tumorigenesis. At ~20 weeks of age, these animals develop primarily thymic lymphomas but also other malignancies such as sarcomas and teratomas (52, 53). In this study, we consistently observed that *p53*$^{-/-}$, *Lck-cre; p53*$^{-/-}$ and *tBrca2*$^{+/-}$*; p53*$^{-/-}$ mice developed T- or B-cell lymphomas and/or other tumors (including osteosarcomas or other high grade malignancies; data not shown). The median tumor-free survival values for these animals were similar: T_{50} = 139.5 days for *p53*$^{-/-}$ mice, T_{50} = 155.5 for *Lck-Cre; p53*$^{-/-}$ mice, and T_{50} = 149.5 days for *tBrca2*$^{+/-}$*; p53*$^{-/-}$ mice (Fig. 7). In

Table 1 *Cytogenetic changes in activated Brca2 mutant or Brca2, p53 double-mutant T cells compared with controls*

Genotype	Metaphases analyzed	Chromosomes per metaphase[a]	Aneuploid cells (%)	Tri-radial-like structures	Chromatid breaks	Chromosome breaks	Fragments[b]	Other aberrations[c]	Mean number of aberrations
WT	100	39.99 ± 0.1 (39–40)	1 (1.0)	0	0	0	2 (0.03/cell)	0	0.03
lck-cre	100	39.99 ± 0.1 (39–40)	1 (1.0)	0	0	1 (0.01/cell)	3 (0.03/cell)	0	0.04
tBrca2$^{+/-}$	50	39.9 ± 0.5 (37–40)	3 (6.0)	0	0	0	4 (0.08/cell)	0	0.08
tBrca2$^{-/-}$	256	39.82 ± 0.5 (38–40)	37 (14.5)	6 (0.02/cell)	13 (0.05/cell)	6 (0.02/cell)	23 (0.09/cell)	3 (0.01/cell)	0.19
p53$^{-/-}$	100	39.8 ± 0.9 (36–41)	10 (10.0)	0	5 (0.05/cell)	2 (0.02/cell)	11 (0.11/cell)	3 (0.03/cell)	0.21
tBrca2$^{-/-}$*; p53*$^{-/-}$	100	38.9 ± 3.0 (23–43)	37 (37.0)	12 (0.12/cell)	28 (0.28/cell)	6 (0.06/cell)	43 (0.43/cell)	15 (0.15/cell)	1.04

[a] Chromosomes per metaphase presented as mean ± SD, and range of chromosome numbers presented in parentheses.
[b] Includes centric, acentric fragments, and interstitial deletions.
[c] Includes translocations and dicentrics.

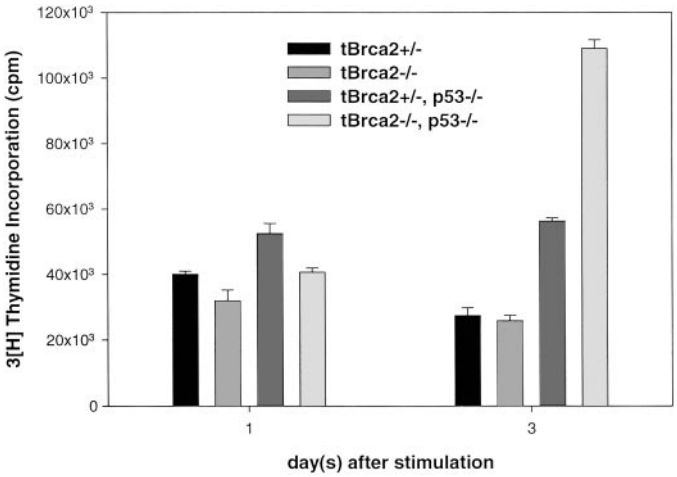

Fig. 6. Increased cell number of activated *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells. Splenocytes from mice of the indicated genotypes were stimulated with anti-CD3ϵ, anti-CD28, and IL-2 as described in "Materials and Methods." At 24 or 72 h after stimulation, the cells were assessed for their proliferative capacity by ^{3}[H]thymidine incorporation. Results shown are the mean ^{3}[H]thymidine incorporation of three cultures/genotype in one representative experiment. Three independent experiments using a total of 5 double-mutant mice were performed; *bars*, ±SD.

contrast, the median tumor-free survival value for *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice was T_{50} = 128.5 days (Fig. 7), and 9 of 10 of these animals developed T-cell lymphomas (one developed an angiosarcoma; Table 2). Using a log-rank test, we can demonstrate that *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice have an accelerated onset in tumorigenesis when compared with *Lck-cre; p53*$^{-/-}$ mice (P = 0.0487) but not when compared with *p53*$^{-/-}$ mice (P = 0.1608) or *tBrca2*$^{+/-}$*; p53*$^{-/-}$ mice (P = 0.0517). Nevertheless, 90% of our *p53*$^{-/-}$ mice succumbed to T-cell lymphoma in the absence of Brca2, whereas other studies have demonstrated that ~70% of p53-null mice develop lymphomas (mostly of thymic origin; Refs. 52, 53). Southern analyses of genomic DNA from T-cell tumors of all 9 *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice revealed that the majority of tumor cells carried the *Brca2*Δ^{9-10} allele (data not shown), implying that oncogenesis in this case was driven by the loss of both p53 and Brca2. Thus, based on our results, it appears that mutations of *Brca2* and *p53* may act synergistically to drive the cancerous transformation of mouse T-lineage cells.

DISCUSSION

A complete deficiency of the breast cancer susceptibility gene *Brca2* is lethal at gastrulation in mice (7, 11, 13). In an attempt to clarify the function of the *Brca2* gene product in adult animals, we used a conditional gene-targeting approach in T cells. The T-lymphocyte lineage is a unique tissue with a highly regulated system for governing cell growth, differentiation, and apoptosis. Proper development of thymocytes requires the rearrangement of both the TCR-α and TCR-β genes, events which involve the formation of DNA DSB and NHEJ of V, D, and J gene segments (reviewed in Ref. 54). Several lines of evidence have implicated Brca2 in the repair of DNA DSB, prompting us to study thymocyte development in the absence of Brca2. However, lymphoid tissues from T cell-specific Brca2 mutants showed normal numbers and distribution of various T-lineage subsets, and mature T cells expressed normal TCR-α and -β chains on the cell surface. Therefore, our results indicate that Brca2 is not required for normal T-lineage development in mice. Another interesting finding of this study was that, whereas activated Brca2-deficient T cells proliferate normally in response to stimulation, they have a higher level of spontaneous cell death. Normal levels of spontaneous apoptosis are restored when p53 is also absent. In addition, we showed that Brca2-deficient T cells have an increased incidence of chromosomal aberrations compared with controls, and that the frequency of these anomalies rises significantly when both Brca2 and p53 are lost.

Mammalian cells can repair DNA DSB using HDR, NHEJ, or SSA. Whereas the NHEJ and SSA pathways are error-prone, HDR produces error-free repair products. The relative contribution of each pathway to the repair of endogenously or exogenously induced DNA DSB depends on tissue type (early developmental or germ line tissues *versus* somatic tissues), cell cycle phase (HDR is predominant in S phase), and the fidelity of the repair required by the cell at risk (error-free repair with HDR *versus* error-prone repair with NHEJ; Ref. 55). Both activated *tBrca2*$^{-/-}$ T cells and MEFs with the Brca2 truncation show an increased number of spontaneous chromatid breaks and tri- and quadri-radial chromosomes, anomalies which are collectively termed "chromatid-type errors" (28, 32, 56). Chromatid-type errors are thought to be repaired almost exclusively by the HDR pathway. Therefore, the occurrence of chromatid-type errors in Brca2-deficient cells supports a role for BRCA2 in HDR of spontaneous DNA lesions. Such a role has been postulated previously for BRCA2, particularly in situations involving the reactivation of stalled replications (25, 56). The observation that proliferating *tBrca2*$^{-/-}$ T cells (which are rapidly replicating their DNA) die spontaneously in culture suggests that the accumulation of chromatid-type errors activates a checkpoint that induces apoptosis. However, the loss of Brca2 does not cause T cells to become hypersensitive to exogenous DNA-damaging stress, in contrast to a report on embryonic cells from the Brca2 truncation mutant (57). Brca2 truncation MEFs also exhibit increased spontaneous mutations but become hypersensitive to IR-induced apoptosis (28). It may be that, at least in mouse T lymphocytes, distinct mechanisms are used to repair endogenously and exogenously induced DSB, whereas the same mechanism repairs both types of lesions in MEFs.

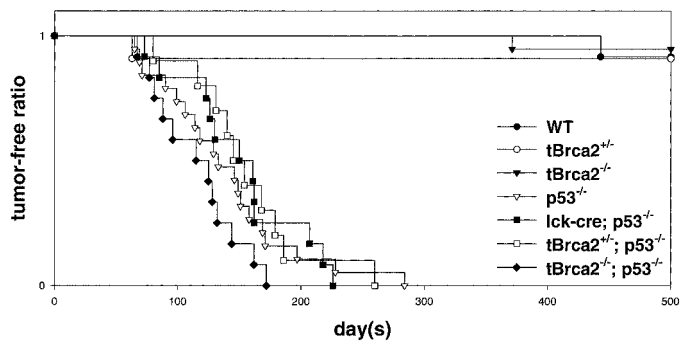

Fig. 7. Tumor-free survival of *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice. Kaplan-Meyer analysis of the tumor-free survival of WT, *tBrca2*$^{+/-}$, and *tBrca2*$^{-/-}$ mice with or without p53. Number of mice of each genotype: WT, n = 12. *tBrca2*$^{+/-}$, n = 11. *tBrca2*$^{-/-}$, n = 19. *p53*$^{-/-}$, n = 18. *Lck-Cre; p53*$^{-/-}$, n = 12. *tBrca2*$^{+/-}$*; p53*$^{-/-}$, n = 10. *tBrca2*$^{-/-}$*; p53*$^{-/-}$, n = 10.

Table 2 *Age of onset and tumor spectrum of tBrca2*$^{-/-}$*, p53*$^{-/-}$ *mice*

Mouse no.	Age (days)	Location of tumor	Type of tumor
1	67	Thymus	T-cell lymphoma
2	77	Thymus	T-cell lymphoma
3	96	Thymus and spleen	T-cell lymphoma
4	115	Thymus and lymph nodes	T-cell lymphoma
5	125	Thymus (involving spleen, liver and lungs)	T-cell lymphoma
6	132	Thymus	T-cell lymphoma
7	144	Thymus	T-cell lymphoma
8	149	Arm	Angiosarcoma
9	157	Thymus	T-cell lymphoma
10	162	Neck	T-cell lymphoma

In contrast to the HDR system, NHEJ repair is intact in at least some Brca2-deficient malignant cell lines (58) and in the fetal liver cells of Brca2 truncation mice (28). Furthermore, in ES cells of the Brca2 truncation mutant, SSA activity is actually increased (59). This latter result provokes the notion that Brca2 deficiency can lead to incorrect routing of DSB processing down inappropriate pathways (16). Our own preliminary data using the single cell electrophoresis Comet assay (60) suggest that *tBrca2*$^{-/-}$ thymocytes subjected to IR have intact DNA SSB rejoining, but exhibit a mild defect in DNA DSB rejoining at 24 h after treatment (data not shown). This observation is consistent with the defect in the repair of endogenous DNA DSB documented in our chromosomal analyses.

Our data have also provided clues to the association between Brca2 and p53 signaling in response to DNA damage in mouse T cells. Whereas *tBrca2*$^{-/-}$ T cells accumulate chromosomal aberrations but retain a normal proliferative potential, *tBrca2*$^{-/-}$*; p53*$^{-/-}$ T cells showed an increase in the frequency of genomic anomalies. We hypothesize that the loss of Brca2 alone in T cells allows chromosomal aberrations to accumulate until a threshold number is reached that induces signaling for p53-mediated apoptosis. Overall cell numbers are thus maintained at a normal level. This theory is supported by the observation that loss of Brca2 in T cells results in an increase in spontaneous apoptosis that does not occur when p53 is lost simultaneously. In the absence of p53 alone, signaling for cell cycle arrest is abrogated so that DNA damage repair cannot initiate. Chromosomal aberrations thus accumulate even when the DNA repair machinery itself is functional. When both Brca2 and p53 are lost, DNA damage can induce neither arrest nor apoptosis. As a result, chromosomal breaks accumulate at an exponential rate. This may explain why T-cell lymphomas occurred at a slightly greater incidence in *tBrca2*$^{-/-}$*; p53*$^{-/-}$ mice compared with *p53*$^{-/-}$ mice.

Similar to *tBrca2*$^{-/-}$ mice, mice with Brca2 truncation also demonstrate normal recombination of *IgH* locus and normal induction of apoptosis in response to IR of lymphoid progenitor cells (28). However, these animals die of thymic lymphomas within the first few months of life, whereas our *tBrca2*$^{-/-}$ mice have survived normally for >18 months. This disparity between the two mouse models may be related to differences in their targeted mutations. Although not confirmed by Western blotting, it is believed that a shortened Brca2 protein of about half the WT size is expressed in mice with the Brca2 truncation. This protein probably encompasses some or most of the eight BRC repeats encoded by exon 11, including the region that binds Rad 51 (19, 20). Because γ-irradiated cells harboring one of these mutations still exhibit Rad 51 focus formation (albeit at a diminished level; Ref. 32), the truncated Brca2 protein may retain partial function and be able to bind to Rad51. A lack of an antimouse Brca2 antibody has precluded us from investigating whether deleting exons 9 and 10 creates a functional null mutation or a hypomorphic mutation with exons 1–8 expressed. Nevertheless, our *Brca2*$^{fl9-10/fl9-10}$ mice succumbed to mammary carcinomas after a long latency when crossed to *MMTV-Cre* or *Wap-Cre* transgenic line (61).[4] This observation has been made previously by another group, which also generated mammary epithelium-specific Brca2 mutants (35), suggesting that deletion of exons 9 and 10 does generate a functional null mutation of *Brca2*.

Mice in which Brca1 is deleted specifically in T cells (*tBrca1*$^{-/-}$ mice) have been generated (39). Spontaneous apoptosis is increased in *tBrca1*$^{-/-}$ thymocytes and the cell cycle is blocked in peripheral *tBrca1*$^{-/-}$ T cells. Given the similarity of their expression profiles, it is surprising that Brca1, but not Brca2, is required for T-lineage development. BRCA1 has been implicated as a scaffolding protein in the "BRCA1-associated genome surveillance complex" (62), which contains several other major DNA damage repair proteins. Interestingly, BRCA2 is not found in this complex, implying that Brca2 may play a distinct role from Brca1 in the DNA damage repair machinery, at least in murine T cells. On the other hand, the loss of either Brca1 or Brca2 in mouse epithelial gland results in cancerous transformation of that tissue after a long latency (35, 63). Like the loss of p53 in *tBrca2*$^{-/-}$ mice, the loss of both p53 and Brca1 or Brca2 accelerates the onset of tumorigenesis in mammary epithelial models (36, 63). However, how the loss of BRCA1 or BRCA2 leads to human breast cancer is still largely unknown. Although the contribution of mutations of the *BRCA* genes to human lymphomagenesis has not been investigated, certain mutations of *Rad54* (64) and *Rad54B* (65), genes which also code for proteins involved in homologous recombination, have been found in human lymphomas. Genomic instability and deregulated apoptosis of T cells resulting from loss of Brca2 and p53 as illustrated in this study, and the development of thymic lymphomas in Brca2 truncation mutants, should spur the study of *BRCA* mutations in human lymphomas.

In conclusion, our study has provided insight into the pivotal role of Brca2 in repairing DNA during replication in mouse T lymphocytes, and additionally supports the existence of cooperation between Brca2 and p53 mutations in promoting tumorigenesis.

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Loss of Brca2 and p53 Synergistically Promotes Genomic Instability and Deregulation of T-cell Apoptosis

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