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A Targeted DNA-PKcs-Null Mutation Reveals DNA-PK-Independent Functions for KU in V(D)J Recombination

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Summary

The DNA-dependent protein kinase (DNA-PK) consists of Ku70, Ku80, and a large catalytic subunit, DNA-PKcs. Targeted inactivation of the Ku70 or Ku80 genes results in elevated ionizing radiation (IR) sensitivity and inability to perform both V(D)J coding-end and signal (RS)-end joining in cells, with severe growth retardation plus immunodeficiency in mice. In contrast, we now demonstrate that DNA-PKcs-null mice generated by gene-targeted mutation, while also severely immunodeficient, exhibit no growth retardation. Furthermore, DNA-PKcs-null cells are blocked for V(D)J coding-end joining, but retain normal RS-end joining. Finally, while DNA-PK-null fibroblasts exhibited increased IR sensitivity, DNA-PKcs-deficient ES cells did not. We conclude that Ku70 and Ku80 may have functions in V(D)J recombination and DNA repair that are independent of DNA-PKcs.

Introduction

Efficient repair of DNA double-strand breaks (DSBs) is crucial for cells to maintain genomic integrity. DSBs are induced by agents such as ionizing radiation (IR) or created during physiological processes such as V(D)J recombination. V(D)J recombination is the process by which antigen receptor variable region genes are assembled from component V, D, and J segments in differentiating lymphocytes (Tonegawa, 1983). This site-specific recombination reaction is initiated by proteins encoded by the lymphocyte-specific recombination activating genes (*RAG1* and *RAG2*), which cleave two participating variable region segments from their flanking recombination signal (RS) sequences to generate blunt RS ends and covalently sealed hairpin coding ends (Schatz et al., 1989; Oettinger et al., 1990; Gellert, 1992). Subsequently, RS ends are precisely ligated to each other, while coding hairpins are opened and joined in a reaction that often results in loss or addition of nucleotides.

The joining phase of V(D)J recombination requires generally expressed proteins that are used in DNA double-strand break repair (DSBR) (reviewed in Taccioli et al., 1992; Weaver, 1995). A subgroup of mutant rodent cell lines that are IR hypersensitive are also defective for V(D)J recombination (Taccioli et al., 1992, 1993, 1994a; Pergola et al., 1993; Smider et al., 1994). Studies of these cell lines and cells from homozygous *scid* (SCID) mice led to the identification of four proteins required for DSBR, including the three subunits of the DNA-dependent protein kinase complex (DNA-PK) and XRCC4 (reviewed in Jeggo, 1997). DNA-PK is a serine-threonine protein kinase consisting of a 465 kDa catalytic subunit (DNA-PKcs) and a DNA-binding subunit comprised of the Ku70 and Ku80 heterodimer (Lees-Miller et al., 1990; Gottlieb and Jackson, 1993). In vitro studies indicated that Ku binds to DSBs and associates with DNA-PKcs, stimulating the kinase activity (Gottlieb and Jackson, 1993). However, recent findings have revealed that DNA-PKcs may bind double-strand DNA ends and become catalytically activated in the absence of Ku (Yaneva et al., 1997; Hammarsten and Chu, 1998). DNA-PKcs belongs to a subfamily of phosphatidyl inositol (PI) 3–related kinases (PI3K) whose members, such as ATM, are very large proteins that are involved in cell cycle progression control in response to DNA damage (Hartley et al., 1995).

Targeted disruption of Ku70 and Ku80 genes resulted in defects in DNA end joining that manifest as IR sensitivity and inability to rejoin both coding and RS ends during V(D)J recombination (Nussenzweig et al., 1996; Zhu et al., 1996; Gu et al., 1997a, 1997b). The V(D)J recombination defect of Ku70- or Ku80-deficient mice leads to a severe combined immune deficiency. Ku70- or Ku80-deficient mice and cells also have severe growth defects (Nussenzweig et al., 1996; Gu et al., 1997b). The effects of several nontargeted mutations in the mouse and human DNA-PKcs genes have been examined (Bosma et al., 1983; Taccioli et al., 1994a; Lees-Miller et al., 1995; Jhappan et al., 1997; Errami et al., 1998; Fukumura et al., 1998). Of these, the most well-characterized is the murine *scid* mutation, which, like Ku-inactivating mutations, causes a severe combined immune deficiency when homozygous. The *scid* mutation is a missense mutation that results in an 83 amino acid truncation after the C-terminal PI3K homology region of DNA-PKcs and leads to diminished expression of the DNA-PKcs protein (Peterson et al., 1995; Blunt et al., 1996; Danska et al., 1996). The Scid mutation, like the analogous V-3 hamster cell mutation, results in cellular IR sensitivity and impairs V(D)J recombination. However, these mutations only block V(D)J coding end joining and, in contrast to Ku-inactivating mutations, do not markedly affect RS joining (Hendrickson et al., 1988; Lieber et al., 1988; Malynn et al., 1988; Lieber et al., 1997). Furthermore, unlike Ku-deficient mice, SCID mice exhibit no growth defects (reviewed in Jeggo, 1997).

The more severe phenotypes of the Ku-inactivating mutations could be due either to DNA-PKcs-independent Ku functions or reflect residual DNA-PKcs protein in SCID or V3 cells that is sufficient to carry out some

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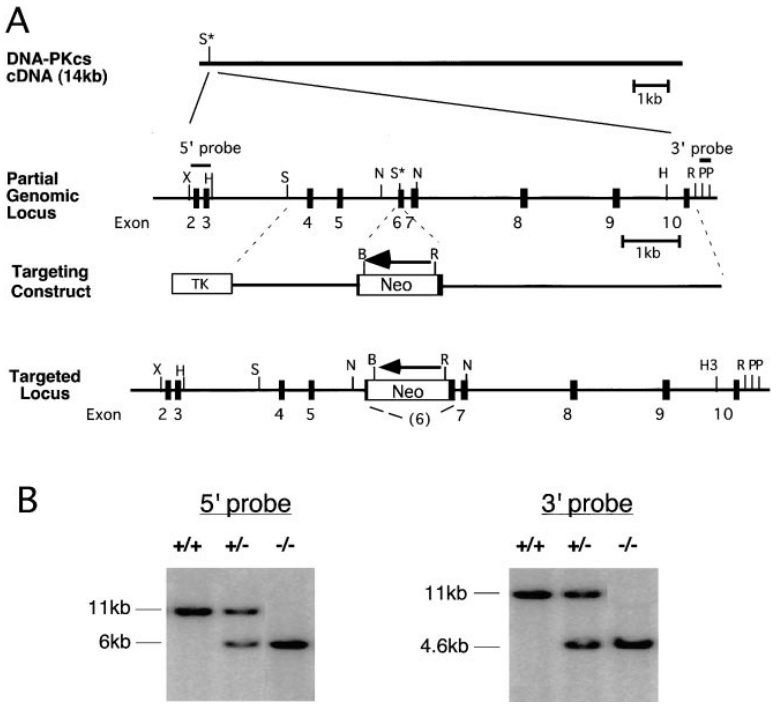

Figure 1. Targeted Disruption of the DNA-PKcs Gene in Mouse ES Cells

(A) Schematic diagram of the mouse DNA-PKcs cDNA, partial genomic map of the 5′ region of the gene, the targeting construct, and targeted locus. Numbered solid boxes represent exons. Positions of the 5′ and 3′ probes used to screen for targeting events are shown. The arrow above *neo*^{r} (G418 resistance gene) indicates its transcriptional orientation. TK, thymidine kinase. Selected restriction sites: X, XbaI; H, HindIII; S, SacI; N, NcoI; R, EcoRI; P, PstI. S* is the SacI site in exon 6.

(B) Representative Southern blot analyses used to identify DNA-PKcs$^{+/N}$ and DNA-PKcs$^{N/N}$ ES cell clones. DNAs from ES clones were digested with BamHI and hybridized with the 5′ or 3′ probes that are indicated in (A). The germline BamHI band is 10.5 kb with both probes, and the targeted band is 6 kb (5′ probe) or 4.6 kb (3′ probe).

(Ku-dependent) activities. The latter explanation has received strong support from several different studies. The first was the characterization of an equine *scid* mutation that leads to a truncated and unstable DNA-PKcs gene product lacking the PI3K homology domain (Shin et al., 1997). This mutation appeared to impair V(D)J joining of both coding and RS ends (Wiler et al., 1995). Additional support for the requirement of DNA-PK kinase activity for both coding and RS joining came from characterization of a murine DNA-PKcs mutation (termed SX9) that was identified in IR-sensitive murine cells and that resulted in severely impaired joining of both coding and RS ends (Fukumura et al., 1998). The SX9 mutation, however, permits generation of significant levels of a mutant DNA-PKcs protein that harbors a leucine to proline substitution upstream of the PI3K homology (Peterson et al., 1997; Fukumura et al., 1998). Additional evidence supporting a requirment for DNA-PKcs in RS joining has come from studies of the XR-C1 CHO line, which has defective DNA-PKcs expression (for undefined reasons) and also is impaired in formation of both RS and coding joins (Errami et al., 1998).

Thus, the prevailing view clearly is that the difference between the Ku-deficient phenotypes and the Scid (or V-3) phenotypes results from the generation of a partially functional protein in Scid cells. However, none of the characterized mutations have been definitively shown to represent null mutations, and the potential existence of mutant DNA-PKcs protein raises the possibility of other interpretations, such as dominant negative effects. To unequivocally address the possibility of DNA-PKcs-independent functions for Ku and to further explore DNA-PKcs function, we have used a gene-targeting strategy to generate a mutation that abrogates DNA-PKcs expression. Our analyses of DNA-PKcs-deficient cells and mice demonstrate a phenotype highly reminiscent of the Scid phenotype and, therefore, support the unanticipated conclusion that Ku70 and Ku80 have roles in V(D)J recombination and DNA repair that are independent of DNA-PKcs.

Results

Generation of DNA-PKcs-Deficient ES Cells

To gain more insight into the function of DNA-PKcs and its functional relationship with Ku proteins in DSBR and V(D)J recombination, we employed a gene targeting strategy to generate embryonic stem (ES) cells that harbored a mutation that abrogates DNA-PKcs expression. For this purpose, we inserted a neomycin-resistance gene (*neo*^{r}) into exon 6 in the opposite transcriptional orientation from that of the DNA-PKcs gene (Figure 1A; Fujimori et al., 1997). Any potential transcript from the targeted allele would bear the *neo*^{r} gene insertion near the start of its coding sequence, precluding translation of a functional DNA-PKcs protein. Multiple heterozygous mutant (DNA-PKcs$^{+/N}$) TC-1 ES cell clones were obtained as judged by Southern blot analyses of BamHI-digested genomic DNAs isolated from these clones (a representative result is shown in Figure 1B). To exclude any undesired random integration of the *neo*^{r} gene, EcoRI-digested DNAs were probed with a *neo*^{r} fragment, confirming that all selected clones contained only the desired targeting event (data not shown).

We used DNA-PKcs$^{+/N}$ ES cells to transmit this mutation into the mouse germline. In marked contrast to Ku-deficient mice, but similar to SCID mice, DNA-PKcs$^{N/N}$ mice showed no evidence of growth retardation as compared to their wild-type and heterozygous littermates (data not shown). For additional analyses, we also used two independent DNA-PKcs$^{+/N}$ ES clones to generate homozygous mutant (DNA-PKcs$^{N/N}$) clones by the increased G418 selection protocol (Gu et al., 1997a). Several DNA-PKcs$^{N/N}$ clones were obtained as judged by

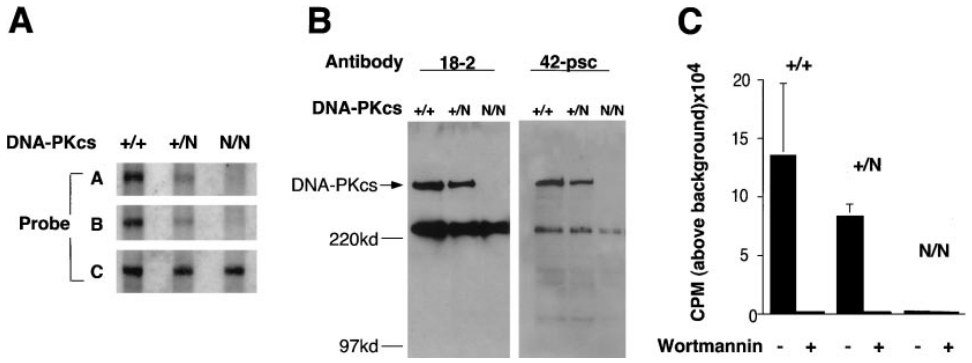

Figure 2. Analysis of DNA-PKcs Expression in DNA-PK$^{N/N}$ ES Cells

(A) Northern blot analyses of total RNA (30 μg) isolated from wild-type (TC-1), DNA-PKcs$^{+/N}$, and DNA-PKcs$^{N/N}$ (2–35–2) ES cell clones. Triplicate bolts were probed with (A) a 5′ mouse DNA-PKcs cDNA fragment (200 bp downstream of the targeted insertion site), (B) a 3′ fragment (encoding PI3K homology region), and (C) a mouse ATM cDNA fragment (encoding PI3K homology region) to control for the quality and quantity of RNA used. (B) Western blot analyses of cell lysates from the same cell lines above. Cell lysates (60 μg) were fractionated on 5% SDS-PAGE, transferred to PVDF membrane, and probed with anti-DNA-PKcs antibodies (18–2 or 42-psc). (C) DNA-dependent protein kinase activity from whole-cell extracts of the same cell lines above. Presence or absence of p53 peptide or the DNA-PK inhibitor wortmannin in reactions was used to validate DNA-PK activity (Lees-Miller et al., 1992; Hartley et al., 1995). For DNA-PKcs$^{N/N}$ extracts, the same background level was observed in the presence or absence of the peptide substrate and with or without wortmannin.

loss of the germline 10 kb band on the Southern analysis (Figure 1B). DNA-PKcs$^{N/N}$ clones were viable and showed no obvious differences in growth properties as compared to their wild-type or heterozygous mutant counterparts (data not shown). Three of these clones were subcloned to ensure clonal homogeneity, and these subclones were used for subsequent studies.

Absence of DNA-PKcs Expression in DNA-PKcs$^{N/N}$ ES Cells

We characterized the DNA-PKcs$^{N/N}$ and control ES cells for expression of DNA-PKcs RNA, protein, and DNA-PK activity. Northern blotting of RNA isolated from DNA-PKcs$^{N/N}$ cells failed to detect authentic DNA-PKcs transcripts with probes from 5′ and 3′ regions of the DNA-PKcs cDNA (Figure 2A, probes A and B), whereas the large *ATM* gene transcript was readily observed in all samples (Figure 2A, probe C). However, low levels of transcripts derived from the 5′ or 3′ portions of the DNA-PKcs gene were detectable by RT–PCR (data not shown). Therefore, to further confirm that the targeted mutation inactivated expression of DNA-PKcs, we assayed for DNA-PKcs protein in lysates from wild-type, DNA-PKcs$^{+/N}$ and DNA-PKcs$^{N/N}$ ES cells by Western blotting using two separate monoclonal anti–human DNA-PKcs antibodies (18–2 and 42-psc; Carter et al., 1990) that strongly cross-react with murine DNA-PKcs. The 18–2 antibody recognizes an epitope within the N-terminal 240 kDa of DNA-PKcs, whereas 42-psc reacts with an epitope in the C-terminal 150 kDa (Casciola-Rosen et al., 1995; Song et al., 1996). DNA-PKcs protein was readily observable in wild-type and DNA-PKcs$^{+/N}$ extracts with either antibody; however, both failed to detect either full-length or any aberrantly sized DNA-PKcs polypeptides in DNA-PKcs$^{N/N}$ lysates (Figure 2B). A 220 kDa protein, not derived from DNA-PKcs (Danska et al., 1996), cross-reacts with both antibodies in all extracts and serves as a useful control. As expected from earlier studies, similar Northern and Western blotting assays of SCID pre-B cells revealed detectable DNA-PKcs transcripts and protein (data not shown). Finally, we assayed whole-cell extracts from normal and mutant cells for DNA-PK activity as determined by the specific stimulation of p53 peptide phosphorylation (Hartley et al., 1995; Danska et al., 1996; Jin et al., 1997). We found that DNA-PKcs$^{N/N}$ extracts had no DNA-PK activity above background, as compared to DNA-PKcs$^{+/+}$ extracts that yielded a 6- to 10-fold stimulation (Figure 2C). Based on these data, we conclude that DNA-PKcs$^{N/N}$ cells are null mutants for DNA-PKcs expression.

DNA-PKcs$^{N/N}$ Mice Have a SCID Phenotype

In SCID mice, both B and T lymphocyte development is arrested at an early progenitor stage due to the impairment in V(D)J coding end joining and the resulting failure to produce functional immunoglobulin (Ig) and T cell receptor (TCR) chains required to drive differentiation (Bosma et al., 1983, 1988). To assess the effect of the targeted DNA-PKcs$^{N/N}$ mutation on lymphocyte development in vivo, we examined both primary and peripheral lymphoid organs in DNA-PKcs$^{N/N}$ mice. The total number of thymocytes from DNA-PKcs$^{N/N}$ thymi were dramatically reduced (100- to 300-fold) compared to wild-type and heterozygous littermates (data not shown). Flow cytometry analyses of DNA-PKcs$^{N/N}$ thymocytes revealed the presence of only CD4$^-$CD8$^-$ (double negative [DN]) progenitor thymocytes and no cells representing the more mature CD4$^+$CD8$^+$ (double positive [DP]) or CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ (single positive [SP]) stages that are abundant in control thymi (Figure 3A). The DN thymocytes in the DNA-PKcs$^{N/N}$ thymi also stained positive for CD25, a surface marker present on progenitor T cells at the DN stage (Figure 3A), confirming that they were indeed progenitor T cells blocked in the progression from the DN to DP stage. Similarly, in DNA-PKcs$^{N/N}$ bone marrow, B cell development was arrested at the B220$^+$CD43$^+$ progenitor B cell stage (Figure 3B). More differentiated B220$^+$CD43$^-$ pre-B cells or B220$^+$IgM$^+$ B

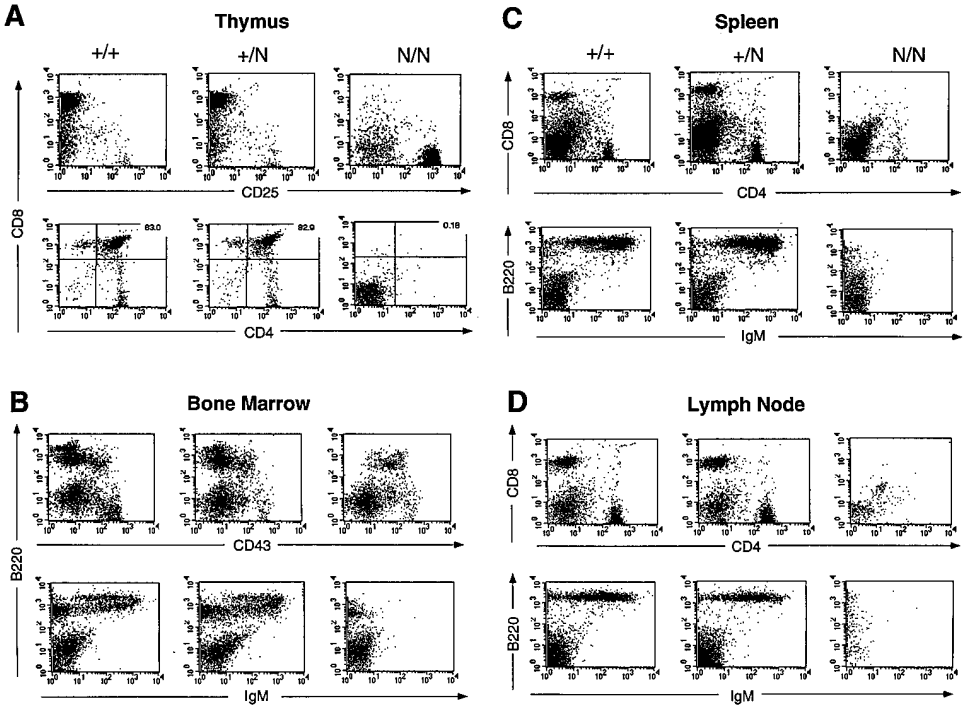

Figure 3. Lymphocyte Development Is Blocked at Early Stage in DNA-PKcs$^{N/N}$ Mice

(A) Block of DNA-PKcs$^{N/N}$ T cell development at CD4$^-$CD8$^-$ (DN) stage. Thymocytes from wild-type, DNA-PKcs$^{+/N}$, and DNA-PKcs$^{N/N}$ mice were triple stained with antibodies to CD4, CD8, and CD25. Expression of these surface markers are shown in dot plots. Percentages of cells in the upper-right quadrant (lower panel) are indicated.

(B) Arrest of B cell development at B220$^+$CD43$^+$ stage in homozygous mutant bone marrow. Bone marrow cells were triple stained with antibodies to B220, CD43, and IgM.

(C) Absence of mature B (B220$^+$IgM$^+$) or T (CD4$^+$ or CD8$^+$) cells in DNA-PKcs$^{N/N}$ spleen. Spleen cell were stained with antibodies to B220 and IgM or CD4 and CD8.

(D) Absence of mature B or T cells in DNA-PKcs$^{N/N}$ lymph nodes. Lymph node cells were stained with antibodies to B220 and IgM or CD4 and CD8.

cells observed in control mice were absent in DNA-PKcs$^{N/N}$ bone marrow (Figure 3B). Analyses of peripheral lymphoid organs (spleens and lymph nodes) again revealed no peripheral mature (SP) T cells or B cells (B220$^+$IgM$^+$) in homozygous mutant mice of up to 6.5 weeks of age (Figures 3C and 3D). We conclude that T and B cell development in DNA-PKcs$^{N/N}$ mice is blocked at an early progenitor stage in which their respective TCR and Ig gene rearrangement processes are initiated. To date, we have not observed any indication of "leakiness" of this phenotype.

Impaired V(D)J Coding Joining but Normal Signal Joining in DNA-PKcs$^{N/N}$ ES Cells and Thymocytes

The severe impairment of lymphocyte development at an early progenitor stage in DNA-PKcs$^{N/N}$ mice suggests a defect in V(D)J recombination. To test this notion, we assayed the ability of DNA-PKcs$^{N/N}$ ES cells to support coding and RS joining by the transient V(D)J recombination assay. In this assay, RAG1 and RAG2 expression vectors, along with V(D)J recombination substrate plasmids for the detection of either coding (pJH290) or RS joining (pJH200), were transfected into wild-type, DNA-PKcs$^{+/N}$, and DNA-PKcs$^{N/N}$ ES cells as previously outlined (Taccioli et al., 1993, 1994b; Gu et al., 1997a). Significantly, DNA-PKcs$^{N/N}$ ES cells performed RS joining nearly as efficiently as wild-type and DNA-PKcs$^{+/N}$ ES cells, and recovered joins were all precise with no losses or additions of nucleotides (Table 1). In contrast, coding join formation was severely impaired in DNA-PKcs$^{N/N}$ ES cells relative to that of wild-type and DNA-PKcs$^{+/N}$ cells (Table 1). The junctions of the few coding joins recovered in DNA-PKcs$^{N/N}$ cells showed abnormally large deletions or long palindromic (P) elements characteristic of SCID or V3 cells (data not shown).

To determine whether the rearrangement of endogenous antigen-receptor gene segments in lymphocytes is affected by the targeted inactivation in vivo, we measured TCRβ gene rearrangement in DNA-PKcs$^{N/N}$ thymi, SCID thymi, and control DNA-PKcs$^{+/N}$ thymi by polymerase chain reaction (PCR) using primers flanking the Vβ8 or Dβ2 and Jβ2 coding segments. We observed Dβ2-Jβ2 joins in DNA-PKcs$^{N/N}$ thymi but at levels markedly reduced compared to controls and similar to those of SCID mice (Figure 4A, left panel). Furthermore, in DNA-PKcs$^{N/N}$ thymi, as in SCID thymi, Vβ8-DJβ2 joins were barely detectable and at greatly reduced levels compared to those of controls (Figure 4A, right panel). To measure the level of endogenous RS joining in DNA-PKcs$^{N/N}$ T cells, we employed a similar PCR approach using primers flanking the RS sequences of Dδ2 and Jδ1. Significantly, the level of Dδ2-Jδ1 RS joins was

Table 1. Analysis of Signal and Coding Join Formation in DNA-PKcs-Deficient ES Cells by Transient V(D)J Recombination Assay

	Signal (pJH200)				Coding (pJH290)		
Cell Line	Amp^RCam^R / Amp^R	Rate (%)	Relative Level	Fidelity (%)	Amp^RCam^R / Amp^R	Rate (%)	Relative Level
Exp. I							
TC-1 (+/+)	905/150000	0.60	100	100 (10/10)	808/128500	0.6	100
2 (+/N)	601/94000	0.64	107	100 (10/10)	279/56000	0.5	80
2-35-2 (N/N)	60/31000	0.20	33	100 (5/5)	6/72000	0.008	1.3
39-10-2 (N/N)	307/72500	0.42	70	100 (5/5)	8/16200	0.005	0.8
Exp. II							
TC-1 (+/+)	260/69000	0.38	100	100 (3/3)	1510/143000	1.0	100
2 (+/N)	125/28500	0.44	115	100 (3/3)	1360/107000	1.3	118
2-35-2 (N/N)	60/57500	0.11	29	100 (6/6)	4/112000	0.003	0.27
39-10-2 (N/N)	370/230000	0.16	42	100 (6/6)	15/137500	0.01	0.9
Exp. III							
TC-1 (+/+)	49/19000	0.29	100	100 (3/3)	441/24800	1.8	100
2 (+/N)	63/21200	0.31	107	100 (3/3)	216/10400	2.1	120
2-35-2 (N/N)	38/17600	0.30	103	100 (5/5)	2/5000	0.04	2
39-10-2 (N/N)	44/18200	0.25	86	100 (5/5)	1/16600	0.007	0.4

Relative levels are normalized to 100% for wild type. Fidelity is expressed as the percentage of recovered RS joins that were susceptible to ApaLI digestion.

comparable between DNA-PKcs$^{N/N}$ and SCID thymocytes (Figure 4B), and most of these joins appeared precise (digestable with ApaLI; data not shown). As unopened hairpin coding ends accumulate in SCID thymocytes, we tested whether this is also true for DNA-PKcs$^{N/N}$ T cells using ligation-mediated PCR approach (LMPCR) (Roth et al., 1992; Zhu and Roth, 1995). A similar accumulation of hairpin coding ends was found in DNAs from SCID and DNA-PKcs$^{N/N}$ thymocytes but was not detected in DNA-PKcs$^{N/+}$ controls (Figure 4C; compare lanes 3, 4, and 5; top band). These coding ends were unopened since they were not detectable in the absence of mung bean nuclease treatment (Figure 4C, lanes 1 and 2).

Together, these studies demonstrate that the effects of the DNA-PKcs null mutation on V(D)J recombination is essentially indistinguishable from those of the SCID mutation and clearly different from that of homozygous Ku inactivating mutations.

DNA-PK$^{N/N}$ Fibroblasts, but Not ES cells, Are Hypersensitive to Ionizing Radiation

The DNA-PKcs-deficient hamster V3 cell line and the human glioma cell line M059J are highly radiosensitive (Taccioli et al., 1994a; Lees-Miller et al., 1995; Allalunis-Turner et al., 1997). Likewise, cells from SCID mice also display IR sensitivity, although the level of sensitivity

Table 2. Comparison of Phenotypes between DNA-PKcs Mutants

Mutation	Protein level	Kinase activity	V(D)J RS joining	V(D)J coding joining	$CD4^+CD8^+$ cells in thymus
Wild type (4128; Leucine zipper; PI3K)	++	++	+	+	++
Murine scid (*4046)	low	–	+	–	low
Equine scid (*3155)	low	–	–	–	N/D
SX9 (*3191 Leu→Pro)	+	–①	–	–	
DNA-PKcs$^{N/N}$ (*53)	–	–	+	–	–

PI3K, phosphatidylinositol 3-kinase homology domain; "*", indicates the position (in terms of amino acid) of mutation; "+", positive/normal; "-", negative/defective; "N/D", undetermined. ①, SX9 mutant protein is able to bind to DNA in the presence of Ku proteins. Solid boxes represent actual DNA-PKcs proteins made in the indicated mutant cells, dashed boxes represent putative full-length polypeptide (undetectable protein expression in mutant cells) showing the position of indicated mutation.

References: (Bosma et al., 1988; Hartley et al., 1995; Peterson et al., 1995; Wiler et al., 1995; Blunt et al., 1996; Danska et al., 1996; Peterson et al., 1997; Shin et al., 1997; Fukumura et al., 1998).

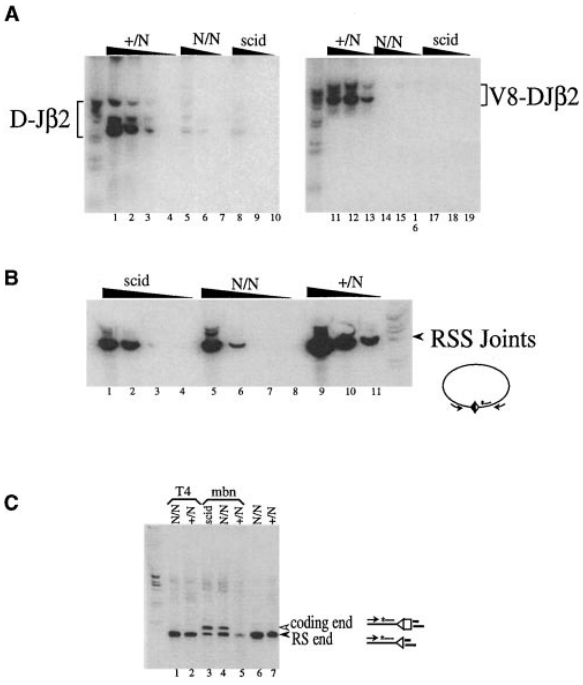

Figure 4. Analysis of V(D)J Recombination Intermediates and Products in DNA-PKcs$^{N/N}$ Thymocyte DNA

(A) Detection of DJ and V-DJ at the TCRβ locus. Left panel, a primer downstream to Jβ2.6 was paired with Dβ2 upstream primer, and PCR products were detected by hybridizing to Jβ2.6-specific oligonucleotides labeled with ^{32}P-ATP. Right panel, Vβ8 primer and Jβ2.6 downstream primer were used to amplify the V-DJ2 rearrangement, and PCR products were detected by hybridizing to the Jβ2.6-specific probe. Thymocyte DNA (100, 10, and 1 ng) was used for PCR amplification. A PCR reaction with no DNA was shown in lane 4 as a negative control.

(B) Detection of circular Dδ2 to Jδ1 RSS joins. Thymocyte DNA samples from DNA-PKcs$^{+/N}$, DNA-PKcs$^{N/N}$, and SCID mice in the amount of 100, 10, and 1 ng were used for PCR amplification. Control reactions with no DNA were shown in lanes 4 and 8. PCR primers and probe were indicated.

(C) LMPCR assay for V(D)J recombination intermediates at the TCRδ locus. Untreated, mung bean nuclease (MBN) or T4 DNA polymerase (T4) treated thymocyte DNA were ligated to the ligation oligonucleotides and PCR amplified using a TCR Dδ2 upstream specific primer (Roth et al., 1993; Zhu and Roth, 1995). DSB at 5′ of Dδ2 generates an RS end at 119 bp; DSB at 3′ of Dδ2 generates a coding end at 134 bp. If a coding end is covalently sealed, it can only be detected in the mung bean nuclease–treated samples. Our data showed that similar amount of RS ends were present in DNA-PKcs$^{+/N}$ and DNA-PKcs$^{N/N}$ mice (compare lanes 1 and 2 with 6 and 7); a slightly reduced level in lane 5 is due to some DNA loss during the nuclease treatment and purification.

Triangles, RS sequences; boxes, coding sequences; lines with arrows, PCR primers; lines with an asterisk, labeled probes; bold lines, ligation oligonucleotides.

varies with cell type (Fulop and Phillips, 1990; Biedermann et al., 1991; Hendrickson et al., 1991). Therefore, to further delineate the effects of the DNA-PKcs null mutation, we used a colony survival assay to assess IR sensitivity of mutant versus normal ES cells. Surprisingly, DNA-PKcs$^{N/N}$ ES cells had the same radiosensitivity as their wild-type or heterozygous mutant counterparts (Figure 5A). As a control for radiosensitivity in ES cell type, Ku70$^{-/-}$ ES cells showed the expected IR hypersensitivity (Figure 5; Gu et al., 1997a). Thus, in contrast to Ku-deficient ES cells, several independent clones of DNA-PKc$^{N/N}$ ES cells are not IR sensitive. To further elucidate the surprising lack of IR hypersensitivity observed in DNA-PKc$^{N/N}$ ES cells, we also analyzed IR sensitivity of fibroblasts derived from DNA-PKcs$^{N/N}$ embryos (MEFs). The DNA-PKcs$^{N/N}$ MEFs showed markedly increased IR sensitivity as compared to their wild-type and heterozygous mutant counterparts (Figure 5B), and their relative sensitivity was similar to that observed for Ku70-deficient MEFs (data not shown). Therefore, although the DNA-PKc$^{N/N}$ ES cells do not show increased IR sensitivity, such sensitivity appears in more differentiated DNA-PK-deficient cell types.

Discussion

Although a substantial amount of data are available from studies on previously characterized DNA-PKcs mutants (summarized in Table 2), interpretation of these studies has been somewhat ambiguous, since most mutants have detectable protein expression and/or the nature of the mutation has not been determined (Peterson et al., 1995; Wiler et al., 1995; Danska et al., 1996; Jhappan et al., 1997; Peterson et al., 1997; Shin et al., 1997; Errami et al., 1998; Fukumura et al., 1998). Our current study circumvents such issues because we have targeted a defined DNA-PKcs mutation that represents a null mutation, as evidenced by the absence of DNA-PKcs protein as detected by separate antibodies to C-terminal and N-terminal portions of DNA-PKcs (Figure 2). The phenotype of our DNA-PKcs$^{N/N}$ mice is surprisingly similar to the phenotype of classical SCID mice. The only potential difference at this point is the lack of any detectable leakiness in the block in B and T cell development (even at the level of DP thymocytes) in the DNA-PKcs$^{N/N}$ mice, as opposed to the known leakiness of this phenotype in SCID mice. However, such differences may be attributable to factors such as age and genetic background (Bosma et al., 1988, 1989).

DNA-PKcs Is Not Required for RS Joining during V(D)J Recombination Reaction

Analyses of previous DNA-PKcs mutants have led to a prevailing opinion that the DNA-PKcs is required for RS joining and that the formation of RS joins in SCID or V3 cells may result from the formation of a partially functional protein (reviewed in Jeggo, 1997). In particular, it has been suggested that a kinase-active DNA-PKcs protein is required for RS joining, based on the lack of RS joining in cells harboring the equine *scid* mutation or the newly defined murine SX9 mutation, both of which eliminated kinase activity (Table 2). In this scenario, the normal RS joining observed in *scid* or V3 cells could have resulted from the residual kinase-active DNA-PKcs, since the known *scid* mutation resides downstream of the kinase homology domain and leads to the generation of a low level of DNA-PKcs protein (Blunt et al., 1996; Danska et al., 1996). However, our studies of the targeted null mutation argue strongly against this interpretation. By transient V(D)J recombination assays, DNA-PKcs-null ES cells were only impaired in coding end joining, while RS end joining remained normal (Table 1). Further analyses of in vivo RS

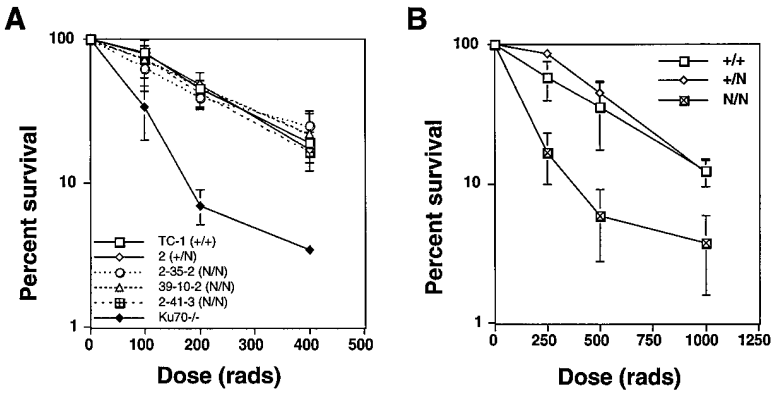

Figure 5. Effect of Ionizing Radiation on DNA-PKcs$^{N/N}$ Fibroblasts and ES Cells

(A) Radioresistance of DNA-PKcs-deficient ES cells. Wild-type (TC-1), DNA-PKcs$^{+/N}$ (2), and three independent DNA-PKcs$^{N/N}$ lines were irradiated by indicated doses of X- or γ-ray and cultured for 7 days before the surviving colonies were stained and counted (Gu et al., 1997a). As an experimental control, Ku70$^{-/-}$ ES (J-1) cells (Gu et al., 1997a) were compared in the assay. IR sensitivity is expressed as the percentage of surviving colonies from irradiated cells over unirradiated control cells.

(B) Survival of γ-ray (doses as indicated) irradiated MEFs (all in their first passage) was measured as a percentage over unirradiated, nonconfluent control cultures 5 days after irradiation. Error bars represent standard deviation from four sets of independent experiments.

joining in thymocytes showed comparable levels of RS joining between DNA-PKcs$^{N/N}$ and the SCID control, which has known ability to join RS ends (Lieber et al., 1988; Figure 4B). Thus, our data strongly support a second interpretation that DNA-PKcs is not required for RS joining. This notion is further supported by findings reported in an accompanying article that a different gene targeted mutation, which specifically deleted the DNA-PKcs kinase domain, also displayed normal RS joining (Taccioli et al., 1998 [this issue of *Immunity*]). Thus, two separate mutations, specifically targeted to different regions of DNA-PKcs, both led to loss of DNA-PKcs expression but had no major effect on RS join formation. Together, these studies strongly argue against the possibility that the retention of RS joining activity in these mutants resulted from some uncharacterized product of the very large DNA-PKcs gene that was not detected by the various anti-sera employed.

Given our current findings, we must seek other explanations for the more severe phenotypes of the DNA-PKcs mutations in equine SCID, SX9 cells, and XR-C1 cells. One interesting possibility is that the impaired RS joining observed in these mutants could have resulted from a dominant negative effect of the mutations. For example, it is possible that these cells generate a stable yet inactive mutant DNA-PKcs protein that inhibits normal Ku function in RS joining, for example, by associating with Ku and/or RS ends. In this context, the mutant DNA-PKcs protein expressed by SX9 cells is stable and able to bind DNA (Peterson et al., 1997). Another possibility is that mutant cell lines such as SX9 or XR-C1, which were selected based on IR hypersensitivity, might have mutations in other genes. However, given the ability to significantly rescue the SX9 phenotype via transfection of a full-length DNA-PKcs cDNA, this possibility seems less likely, at least for that line (Fukumura et al., 1998). In the context of a dominant negative scenario, the ability to rescue SX9 with a wild-type cDNA could be explained if the effects of the dominant negative were countered by the wild-type DNA-PKcs protein. To resolve this issue and further elucidate DNA-PKcs function, it will be quite useful to introduce constructs encoding various mutant forms of the DNA-PKcs proteins into the DNA-PKcs$^{N/N}$ cells and test for their ability to support RS joining.

Differential Involvement of DNA-PKcs and Ku in V(D)J Recombination

Our studies of DNA-PKcs-deficient ES cells clearly demonstrate that Ku has functions in V(D)J recombination and normal development that are distinct from any in the context of a DNA-PK holoenzyme. Loss of Ku expression results in growth defects in mice as well as defective coding and RS end joining in cells (Nussenzweig et al., 1996; Gu et al., 1997a). In contrast, DNA-PKcs-deficient mice are normal in size and DNA-PKcs-deficient cells, while impaired in coding end joining, have normal ability to join RS ends. Therefore, our studies unequivocally demonstrate that RS joining does not require DNA-PK or DNA-PK-dependent phosphorylation of proteins, in contrast to the absolute requirement for Ku proteins in this process. This conclusion also solidifies the notion that the V(D)J coding and RS joining pathways are biochemically distinct.

There are various mechanisms by which Ku proteins may function independently of DNA-PKcs in the RS joining process. Ku alone may protect DNA ends, facilitate their tethering for subsequent processing (Ramsden, 1998), or may help dislodge the RAG postsynaptic complex from the ends (Zhu et al., 1996; Hiom and Gellert, 1998). Ku proteins may also facilitate end joining by unwinding DNA ends through its intrinsic helicase activity (Tuteja et al., 1994) or recruiting as yet undefined proteins required for the process. On the other hand, the function of DNA-PKcs in V(D)J coding end joining may well be mediated through its interaction with Ku. Alternatively, DNA-PKcs might function independently of Ku in this process, as suggested by biochemical studies showing that DNPKcs has weak DNA binding and kinase activity (Yaneva et al., 1997; Hammarsten and Chu, 1998). Among the possible roles for DNA-PK in coding joining is some role, either direct or indirect, in the opening of hairpin ends, as supported by the accumulation of hairpin coding ends in *scid* thymocytes (Roth et al., 1992) and DNA-PKcs$^{N/N}$ thymocytes (this study).

Roles of Ku versus DNA-PKcs in Other Processes

In mammalian cells, DSBR processes are achieved both by DNA end joining and homologous recombination-mediated mechanisms (Liang et al., 1998; reviewed in

Weaver, 1995). The apparently normal IR sensitivity of DNA-PKcs-deficient ES cells was unexpected given the known IR sensitivity of SCID fibroblasts or pre-lymphocyte cell lines. Because we did find IR sensitivity in DNA-PKcs-deficient fibroblasts, it seems possible that ES cells may employ a DNA-PKcs-independent DSBR pathway or a factor redundant with DNA-PKcs for DNA repair that is not principally utilized in other cell types. In yeast, there are two end joining pathways that are operative in different phases of the cell cycle (Moore and Haber, 1996). Thus, the presence of an additional pathway to repair IR-induced DNA damage in ES cells versus certain somatic cell types may reflect cell cycle differences or, given the germ cell nature of ES cells, the presence of repair pathways or factors not active later in embryonic development or adulthood.

It is notable that characterized Ku70-deficient ES cells, unlike DNA-PKcs-deficient ES cells, do show increased IR sensitivity (Gu et al., 1997a). These findings suggest that Ku may also have DNA-PKcs-independent functions in DNA repair, although the apparently differential effect of Ku70 versus DNA-PKcs mutations will need to be confirmed via the generation and complementation of double mutants. Furthermore, the much less severe phenotype of DNA-PKcs$^{N/N}$ mice compared to Ku-deficient mice in terms of growth characteristics indicates a role for Ku that extends beyond V(D)J recombination and its association with DNA-PKcs. Likewise, while Ku-deficient MEFs displayed severe growth retardation and early senescence (Nussenzweig et al., 1996; Gu et al., 1997b), DNA-PKcs$^{N/N}$ MEFs were normal in these aspects (data not shown). Together, these findings also imply that Ku might be involved in proliferation control in DNA-PKcs-independent ways such as regulating the structure of telomeres (Boulton and Jackson, 1996; Tsukamoto et al., 1997; Gravel et al., 1998).

Experimental Procedures

Generation of DNA-PKcs-Deficient ES Cells

Murine DNA-PKcs genomic clones were obtained by screening a 129 strain λ phage genomic library (Strategene) with a 1 kb fragment of 5′ DNA-PKcs cDNA as a probe. Three overlapping clones were characterized that cover the region from exon 2 to exon 10 (Fujimori et al., 1997). A targeting construct was made based on the genomic map with the neomycin-resistance gene (*neo*^{r}) inserted in the SacI site of exon 6. A thymidine kinase (*tk*) gene in the construct served for negative selection of random integration event of the construct.

The construct was linearized and transfected into TC-1 ES cells (kindly provivded by Dr. Philip Leder, Harvard Medical School, Boston, MA) by electroporation. Positive and negative selection of transfectants in media containing G418 (0.4 mg/ml) and ganciclovir (1 μM) was performed as described before (Gu et al., 1997a). Genomic DNA from individual double-resistant (G418 and ganciclovir) clones were digested with BamHI and probed with a 5′ flanking probe (600 bp XbaI–HindIII fragment) on a Southern blot. A 3′ flanking probe (240 bp PstI–PstI fragment) was then used on the same Southern blot to ensure proper homologous recombination at the 3′ end of the targeted region. The presence of a 6.4 kb BamHI band upon hybridization with the 5′ probe and a 4.6 kb band when using the 3′ probe indicated proper targeting of the DNA-PKcs gene. A *neo* gene probe was then hybridized with EcoRI-digested DNA to exclude any undesired integration event of the construct.

To generate ES cells homozygous for the targeted mutation, two independent DNA-PKcs$^{+/N}$ clones, 2 and 39, were further selected for resistance to higher concentrations of G418 in the culturing media as described (Gu et al., 1997a). Using the Southern analyses described above for identifying DNA-PKcs$^{+/N}$ clones, double-knockout clones were identified by loss of the remaining germline 10.5 kb band.

Generation of DNA-PKcs$^{N/N}$ Mice

Heterozygous DNA-PKcs mutant ES cells were injected into C57Bl/6 blastocysts and chimera mice generated as described (Zhou et al., 1995). Male chimeric mice were bred with C57Bl/6 females to transmit the targeted allele to the germline. F1 heterozygotes were then intercrossed to generate homozygous mutant mice.

Northern Blot Analyses

Total RNA was isolated from wild-type (TC-1), DNA-PKcs$^{+/N}$, and DNA-PKcs$^{N/N}$ ES cell clones using Trizol (GIBCO BRL). Total RNA (30 μg) was electroforesed on 1% formaldehyde-agarose gel and transferred to Z-probe membrane (Millipore). Triplicate blots were made for hybridization with three probes. Autoradiography was performed on phosphoimager (Molecular Dynamics) and data processed with Storm software (Molecular Dynamics).

Western Blot Analyses

Cell lysates were prepared from wild-type, heterozygous, and homozygous mutant ES cells in lysis buffer (50 mM Tris [pH8.0], 200 mM NaCl, 2 mM $MgCl_2$, 0.5% NP-40, and 2 mM PMSF plus proteinase inhibitor cocktail [Boehringer Mannheim]). Lysate (60 μg; estimated using Bio-Rad protein assay kit) from each cell type was fractionated on 5% SDS-PAGE, transferred to PVDF membrane (Millipore), and probed with anti-DNA-PKcs antibodies (18–2 or 42-psc, Neomarkers). Detection was performed using ECL+ (Amersham).

DNA-Dependent Protein Kinase Assay

Whole-cell extracts were prepared from wild-type, heterozygous, and homozygous mutant ES cells with whole-cell extract buffer: 20 mM HEPES (pH 8.0), 500 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, and 2 mM PMSF plus proteinase inhibitors cocktail (Boehringer Mannheim). The extracts were tested for DNA-PK activity by absorption of protein onto double-stranded DNA cellulose beads followed by phosphorylation of a p53 peptide substrate with minor modifications (Danska et al., 1996; Jin and Weaver, 1997). Reactions were stopped by 15% acetic acid followed by 10% TCA precipitation prior to binding and washing on phosphocellulose paper. Presence or absence of p53 peptide or the DNA-PK inhibitor wortmannin in reactions was used to validate DNA-PK activity (Lees-Miller et al., 1992; Hartley et al., 1995). For DNA-PKcs$^{N/N}$ extracts, the same background level was observed in the presence or absence of the peptide substrate and with or without wortmannin.

Transient V(D)J Recombination Assay

Assays were performed as described previously (Li et al., 1995; Gu et al., 1997a). Briefly, RAG1 and RAG2 expression vectors along with transient V(D)J recombination substrate plasmids, pJH200 for assaying signal joining or pJH290 for coding joining, were transfected into wild-type, DNA-PKcs$^{+/N}$, and DNA-PKcs$^{N/N}$ ES cells. Rearranged substrate plasmids, which would allow the expression of a chloramphenical resistance gene (Cam^r) when transfected into appropriate bacteria (MC1061), were then recovered from those cells. Bacteria were transformed with the rescued substrate plasmids, and plated on Amp + Cam and Amp plates. The recombination efficiency was determined by the ratio of double-resistance colonies to single-resistance colonies (Amp^r + Cam^r/Amp^r). The fidelity of signal joining was assessed by the susceptibility of recovered joins to ApaLI digestion (Li et al., 1995).

Flow Cytometry Analysis of Lymphocytes Development

Single-cell suspensions were prepared from thymus, bone marrow, spleen, and lymph node from 3- to 6-week-old wild-type, heterozygous, and homozygous DNA-PKcs mutant mice as described (Gu et al., 1997a). About 0.5 million cells were used for each staining, using fluorescence-conjugated antibodies as indicated in Figure 3. Stained cells were analyzed on FACSCalibur (Becton Dickinson), and data were processed with Cellquest software (Becton Dickinson).

Radiation Sensitivity Assay for MEFs and ES Cells

ES cells (passed at least twice in the absence of feeder cells) of wild-type, DNA-PKcs$^{+/N}$, and three independent DNA-PKcs$^{N/N}$ lines (all in TC-1) were irradiated by indicated doses of X- or γ-rays and cultured for 7 days before the surviving colonies were stained and counted (Gu et al., 1997a). IR sensitivity is expressed as the percentage of surviving colonies from irradiated cells over unirradiated control cells.

MEFs were isolated from embryos 14 days after gestation from female partners of intercrosses between DNA-PKcs$^{+/N}$ mice as described (Gu et al., 1997a). Survival assay was performed as described (Patel et al., 1998). Briefly, passage 1 MEFs of each genotype were irradiated with 0, 250, 500, and 1000 rads of γ-irradiation and plated in 6-well plates. After 5 days in culture (before any culture reached confluency), cells were trypsinized and survival was determined by trypan blue exclusion. Percentage of surviving cells exposed to irradiation over unirradiated control cells was plotted against radiation dosage.

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