Ku70 Is Required for DNA Repair but Not for T Cell Antigen Receptor Gene Recombination In Vivo

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Summary
Ku is a complex of two proteins, Ku70 and Ku80, and functions as a heterodimer to bind DNA double-strand breaks (DSB) and activate DNA-dependent protein kinase. The role of the Ku70 subunit in DNA DSB repair, hypersensitivity to ionizing radiation, and V(D)J recombination was examined in mice that lack Ku70 (Ku70−/−). Like Ku80−/− mice, Ku70−/− mice showed a profound deficiency in DNA DSB repair and were proportional dwarfs. Surprisingly, in contrast to Ku80−/− mice in which both T and B lymphocyte development were arrested at an early stage, lack of Ku70 was compatible with T cell receptor gene recombination and the development of mature CD4+CD8− and CD4−CD8+ T cells. Our data shows, for the first time, that Ku70 plays an essential role in DNA DSB repair, but is not required for TCR V(D)J recombination. These results suggest that distinct but overlapping repair pathways may mediate DNA DSB repair and V(D)J recombination.

Two distinct processes involving DNA double-strand breaks (DSB)1 have been identified in mammalian cells: the repair of DNA damage induced by ionizing radiation and V(D)J recombination during T and B cell development. So far, all mammalian cell mutants defective in DNA DSB repair share the common phenotype of hypersensitivity to radiation and impaired ability to undergo V(D)J recombination (1–6). Cell fusion studies using DSB sensitivity to radiation and impaired ability to undergo V(D)J recombination (1–6). Cell fusion studies using DSB repair mutants of human–rodent somatic hybrids have defined four ionizing radiation (IR) complementation groups: IR4, IR5, IR6, and IR7. Genetic and biochemical analyses have revealed that cells of IR5 (e.g., xrs-6) and IR7 (e.g., sld) are defective in components of the DNA-dependent protein kinase (DNA-PK) (2, 7–9). DNA-PK is a serine/threonine kinase comprised of a large catalytic subunit and is an important component of the DNA-PK complex, the function of Ku70 in vivo is hitherto unknown. To define the role of Ku70 in DNA repair and V(D)J recombination, we targeted the Ku70 gene in mice. Ku70 homzygotes exhibit proportional dwarfism, a phenotype of Ku80−/−, but not of sld mice. Absence of Ku70 confers hypersensitivity to ionizing radiation and deficiency in DNA DSB repair, which are characteristics of both Ku80−/− and sld mice. Surprisingly, in contrast to Ku80−/− and sld mice in which both T and B lymphocyte development are arrested at early stage, lack of Ku70 is compatible with T cell receptor gene recombination and the development of mature CD4+CD8− and CD4−CD8+ T cells. Our data, for the first time, provide direct evidence supporting that Ku70 plays an essential role in DNA DSB repair, but is not required for TCR gene recombination. These results suggest that distinct but overlapping repair pathways may mediate DNA DSB repair and V(D)J recombination.

1 Abbreviations used in this paper: AFIGE, asymmetric field inversion gel electrophoresis; CFU-GM, granulocyte/macrophage CFUs; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; ES, embryonic stem; Gy, Gray; IR, ionizing radiation; IVS, intervening sequence; SP, single-positive.
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Materials and Methods

Target Disruption of Ku70 and Generation of Ku70−/− Mice. Mouse genomic Ku70 gene was isolated from a sc5s1 cosmId library constructed from a mouse strain 129 embryonic stem (ES) cell line (21). The replacement vector was constructed using a 1.5 kb 5′-fragment that contains the promoter locus with four GC boxes and exon 1, and an 8-kb EcoR V–EcoR I fragment extending from intron 2 to intron 5 as indicated in Fig. 1 A. Homologous replacement results in a deletion of 336-bp exon 2 including the translational initiation codon.

The targeting vector was linearized with NotI and transfected into C57 ES cells by electroporation using a gene pulser (Bio Rad Labs., Hercules, CA). 300 ES cell clones were screened, and five clones carrying the mutation in Ku70−/− were identified by Southern blotting. Positive ES clones were injected separately into C57BL/6 blastocysts to generate chimeric mice. One clone was successfully transmitted through the germline after chimeras were crossed with C57BL/6 females. Homozygous Ku70−/− mice were generated by crossing Ku70−/− heterozygotes.

The genotype of the mice was first determined by tail PCR analysis which distinguishes endogenous from the targeted allele, and subsequently confirmed by Southern blot analysis. The Ku70 targeted ES cell clones were introduced into CJ7 ES cells by electroporation using a gene pulser (Bio Rad). Cycling conditions were 94°C for 1 min, 64°C for 1 min, 72°C for 1 min (30 cycles), followed by an extension at 72°C for 10 min. Primers HO-2 and HO-4 generated a product of the targeted allele that is 380 bp; primers HO-3 and HO-4 yield a wild-type product of 407 bp.

Western blot analysis and mobility shift assay. To confirm that the disruption of Ku70 produces a null mutation, Ku70 protein expression was measured by Western blotting using polyclonal antiserum against intact mouse Ku70. The lack of Ku70 was also verified by a Ku-DNA end-binding assay (gel mobility shift analysis). Cell extracts were prepared and gel mobility shift assays were performed as described (22). Equal amounts of cellular protein (50 μg) from Ku70−/− (wild type), Ku70+/−, and Ku70−/− mouse embryo fibroblasts were incubated with a 32P-labeled double-stranded oligonucleotide, 5′-GGGCTGAGGGCTGATCCATTA-3′, 0.2 μM (each) deoxynucleoside triphosphate; 1.5 mM MgCl2, and 2.5 U of T4 polynucleotide kinase. Cycling conditions were 94°C for 1 min, 64°C for 1 min, 72°C for 1 min (30 cycles), followed by an extension at 72°C for 10 min. Primers HO-2 and HO-4 generated a 380-bp product, while primers HO-3 and HO-4 yielded a wild-type product of 407 bp.

DNA Preparation and Analysis of V(D)J Recombination Products. To determine whether a null mutation in Ku70 affects the recombination of antigen-receptor genes in T and B lymphocytes, four-week-old mice were analyzed. DNA was extracted from lymph nodes, spleens, and thymuses from 4–6-wk-old mice. DNA from thymus, spleen, and lymph nodes from different mice were used for titration of the antibodies and positive controls. Anti-CD3 and anti-CD19 antibodies were tested in both frozen and paraffin sections of wild-type lymphoid organs and showed the expected specific patterns of staining (data not shown). For negative controls, primary antibodies were substituted with class-matched but unrelated antibodies at the same final working dilutions.

Cell Preparation and Flow Cytometric Analysis. For flow cytometry, single cell suspensions from lymphoid organs of 4–6-wk-old mice were prepared for staining as described previously (19) and analyzed on a FACScan with Cell Quest software (Becton Dickinson, San Jose, CA). Cells were stained with combinations of PE-labeled anti-CD4 and FITC-labeled anti-CD8, or PE-labeled anti-B220 and FITC-labeled anti-CD43, or FITC-anti-lgM and PE-anti-B220 (PharMingen), as needed. Bone marrow cells were harvested from femurs by syringe lavage, and cells from thymus and spleen were prepared by homogenization. Cells were collected and washed in PBS plus 5% FCS and counted using a hemacytometer. Samples from individual mice were analyzed separately. Dead cells were gated out by forward and side scatter properties. Experiments were performed at least three times and yielded consistent results.

Oligonucleotides for probes and PCR primers specific to TCR V(D)J rearrangements and immunoglobulin D-JH and V-DJH rearrangements are as follows. For TCR-α Vβ8–1 rearrangements and immunoglobulin D-JH and V-DJH rearrangements as follows. For TCR-β Vβ8–1 rearrangements (28): Vβ8.1S: 5′-GAGGAAAGGCTGACATTGAGC-3′, Jβ2.6: 5′-GGCTGGTGCACGGCCAGCATGTA-3′, Vβ8 probe: 5′-GGGGTGAAGGTGACATTGAGC-3′, Jβ2.5: 5′-TGAATTCACAAGTGACTTGGCTTCCC-3′, and DR2 probe: 5′-GACAGTTGACATACGAGACCCG-3′.

Oligonucleotides for probes and PCR primers specific to TCR Vβ5–1 rearrangements and immunoglobulin D-JH and V-DJH rearrangements are as follows. For TCR-β Vβ8–1 rearrangements (28): Vβ8.1S: 5′-GAGGAAAGGCTGACATTGAGC-3′, Jβ2.6: 5′-GGCTGGTGCACGGCCAGCATGTA-3′, Vβ8 probe: 5′-GGGGTGAAGGTGACATTGAGC-3′, Jβ2.5: 5′-TGAATTCACAAGTGACTTGGCTTCCC-3′, and DR2 probe: 5′-GACAGTTGACATACGAGACCCG-3′.

Immunohistochchemistry. To determine the pathological changes, histological sections of various organs of Ku70−/−, Ku80−/−, and wild-type littermate mice were prepared and examined as previously described (23). Lymph nodes, spleens, and thymuses from 4–5-wk-old mice were fixed in 10% buffered formalin and embedded in paraffin, or embedded in optimal cutting temperature compound (Sakura Finetek, USA, Incorp., Torrance, CA) and frozen in liquid nitrogen at −70°C. Sections (3 μm) were stained with hematoxylin and eosin, and representative samples were selected for immunohistochemical analysis. Immunophenotyping was performed using an avidin-biotin immunoperoxidase technique (24). Primary antibodies included anti-CD3 (purified rabbit serum, 1:1,000; Dako Corp., Carpinteria, CA), anti-B220 (rat monoclonal, 1:1,000; Pharmingen, San Diego, CA), and anti-CD19 (rat monoclonal, 1:1,000; Pharmingen), and were incubated overnight at 4°C. Samples were subsequently incubated with biotinylated secondary antibodies (Vector Labs., Burlingame, CA) for 30 min (goat anti-rabbit, 1:100; rabbit anti-rat, 1:100), and then with avidin-biotin peroxidase (1:25 dilution; Vector Labs) for 30 min. Diaminobezadine was used as the chromogen and hematoxylin as the counter stain. Wild-type lymphoid organs including thymus, spleen, and lymph nodes from different mice were used for titration of the antibodies and positive controls. Anti-CD3 and anti-CD19 antibodies were tested in both frozen and paraffin sections of wild-type lymphoid organs and showed the expected specific patterns of staining (data not shown). For negative controls, primary antibodies were substituted with class-matched but unrelated antibodies at the same final working dilutions.

Cell Survival Determination. 8–10-wk-old Ku70−/− and Ku80−/− mice and wild-type littermates were used for our studies. Bone marrow cell suspensions were prepared by flushing the femur with MEM supplemented with 15% FCS. The cell suspension was then
counted using a hemacytometer and centrifuged at 1,000 rpm for 12 min. The resulting pellet was resuspended and diluted to ~10^6 cells/ml in MEM plus 15% FCS for further experiments.

To measure the survival of granulocyte-macrophage progenitors, the method of Van Zant et al. (29) was used with minor modifications (30). In brief, α-MEM contained 30% heat-inactivated FCS and 1% bovine serum albumin; in addition, 0.5 ng/ml GM-CSF (R & D Sys. Inc., Minneapolis, MN) was used as a source of colony-stimulating factor. 1 d before each experiment, GM-CSF (R & D Sys. Inc., Minneapolis, MN) was used as a source of colony-stimulating factor. Immediately after radiation exposure, cells were diluted in 2 ml of the above media with 0.3% noble agar and poured over the prepared dishes with 0.5% noble agar underlayer. The cells were then incubated at 37°C with 5% CO₂ and 95–98% humidity. The colonies were counted on day 8 with a dissecting microscope. Macrophage and granulocyte colonies were counted separately and then summed together for survival calculations of granulocyte-macrophage progenitors (granulocytes/macrophages CFUs, CFU-GM). Only colonies containing 50 cells were scored. The colony forming efficiency of CFU-GM was 60–100/10^5 nucleated cells for untreated controls. Surviving fraction was defined as the cloning efficiency of irradiated marrow cells relative to that of untreated controls. All experiments were performed at least twice and yielded consistent results.

Asymmetric Field Inversion Gel Electrophoresis. To determine the rate and extent of DNA DSB repair in Ku-deficient cells after exposure to ionizing radiation, primary embryo fibroblasts derived from Kυ70^-/-, Kυ80^-/- and wild-type littermate mice were used. Mouse embryo fibroblasts from day 13.5 embryos growing in replicate cultures for 3 d in the presence of 0.01 μCi/ml [³H]thymidine (New England Nuclear, Boston, MA) and 2.5 μM cold thymidine were exposed to 40 Gray (Gy) of x-rays and returned to 37°C. At various times thereafter, one dish was removed and trypsinized on ice; single cell suspensions were made and embedded in an agarose plug at a final concentration of 3 × 10^6 cells/ml. Asymmetric field inversion gel electrophoresis (AFIGE) was carried out in 0.5% Seakem agarose (FMC Bioproducts, Rockland, ME; cast in the presence of 0.5 μg/ml ethidium bromide) in 0.5 × TBE (45 mM Tris pH 8.2, 45 mM boric acid, 1 mM EDTA) at 10°C for 40 h by applying cycles of 1.25 V/cm for 900 s in the direction of DNA migration, and 5.0 V/cm for 75 s in the reverse direction as described (31).

Quantification and analysis for DNA DSB present were carried out in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Levels of DNA DSB were quantified by calculating the fraction of activity released from the well into the lane in irradiated and unirradiated samples, which equals the ratio of the radioactivity signal in the lane versus that of the entire sample (well plus lane).

**Results**

Targeted Disruption of Kυ70 Gene. To study the role of Kυ70 in vivo, we generated mice containing a germline disruption of the Kυ70 gene. M urine genomic Kυ70 gene was isolated and a targeting vector was constructed (Fig. 1 A). Homologous replacement results in a deletion of 336-bp exon 2, including the translational initiation codon. Two targeted ES clones carrying the mutation in Kυ70 were injected into C57BL/6 blastocysts to generate chimeric mice. One clone was successfully transplanted through the germline after chimeras were crossed with C57BL/6 females. No obvious defects were observed in Kυ70^-/- heterozygotes, and these Kυ70^-/- mice were subsequently used to generate Kυ70^-/- mice (Fig. 1 B). 25% of the offspring born from Kυ70^-/- × Kυ70^-/- crosses were Kυ70^-/- . Adult Kυ70^-/- mice are fertile, but give reduced litter size (two to four pups) as compared to the Kυ70^-/- or Kυ70^-/- mice (about eight pups).

To confirm that the disruption produced a null mutation, Ku70 protein expression was analyzed by both Western blotting (Fig. 1 C) and a DNA end binding assay (Fig. 1 D). Ku70 immunoreactivity was undetectable (Fig. 1 C), and there was no Ku-DNA-end-binding activity in Kυ70^-/- fibroblasts (Fig. 1 D). The Ku80 subunit of the Ku heterodimer was found, but at much reduced levels (Fig. 1 C), suggesting that the stability of Ku80 is compromised by the absence of Kυ70. These observations are consistent with the finding that the level of Kυ70 was significantly reduced in Kυ80^-/- fibroblasts and Kυ80^-/- ES cells (19). Taken together, these data suggest that the stability of either component of Ku is compromised by the absence of the other.

Newborn Kυ70^-/- mice were 40–60% smaller than their Kυ70^-/- and Kυ70^-/- littermates. During a 5-mo observation period, Kυ70^-/- mice grew and maintained body weight at 40–60% of controls. Thus, Kυ70^-/- mice, like Kυ80^-/- mice, are proportional dwarfs (19).

Development of B Lymphocytes, but Not T Lymphocytes, Is Inactivated in Kυ70^-/- Mice. Examination of vari-
ous organs from Ku70−/− mice showed abnormalities only in the lymphoid system (Fig. 2 A). Spleen and lymph nodes were disproportionately smaller by 5–10-fold relative to controls. In particular, splenic white pulp nodules were significantly reduced. Immunohistochemistry on deparaffinized tissue sections revealed that the splenic white pulp contained cells that stained with anti-CD3 (i.e., CD3-positive T cells), but there were no CD19-positive B cells (Fig. 2 A, k and n).
The Ku70−/− thymus was also disproportionately smaller and contained 50-100-fold fewer lymphocytes than Ku70+/+ littermates (3 × 10^6 in the former versus 2 × 10^8 in the latter; measured in three mice of each genotype). In contrast to the Ku80−/− mice, the Ku70−/− thymus displayed normal appearing cortical-medullary junctions (Fig. 2 A, g and j). Overall, the lymphoid tissues and organs of Ku70−/− mice are somewhat disorganized and much smaller than Ku70+/− mice (Table 1); yet, they are relatively more developed and slightly larger than in Ku80−/− mice.

To further examine the immunological defect in Ku70−/− mice, cells from thymus, bone marrow, and spleen were analyzed using monoclonal antibodies specific for lymphocyte surface markers and flow cytometry (19). Consistent with the immunohistological data, there was a complete block in B cell development at the B220+CD43+ stage in the bone marrow, and there were no mature B cells in the spleen (Fig. 2 B). In contrast, thymocytes developed through the CD4+CD8+ double-positive stage and matured into CD4+CD8− and CD4−CD8+ single-positive (SP) TCR-β-positive cells (Fig. 2, B and C). In six 4-wk-old Ku70−/− mice analyzed, the percentage of CD4+CD8− double-negative thymocytes ranged from 11 to 62%, and the CD4+CD8+ double-positive cells varied from 35 to 73%. CD4+CD8− (1-11%) and CD4+CD8+ (1-3%) SP cells were also detected in the thymus. Furthermore, CD4+ CD8− or CD4−CD8+ SP T cells were found in the spleen in 67% of the mice studied (Fig. 2 B), which expressed surface TCR-β (Fig. 2 C) and CD3 (data not shown). Thus, in contrast to the early arrest of both T and B cell development in Ku80−/− mice (Fig. 2 B), lack of Ku70 is compatible with the maturation of T cells.

Table 1. Lymphoid Cellularity of Ku70−/− Mice

<table>
<thead>
<tr>
<th>Tissue and genotype</th>
<th>Cell content (x 10^6)</th>
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<tr>
<td></td>
<td>Total B220^+ CD4^+CD8^+</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
</tr>
<tr>
<td>wild type (n = 4)</td>
<td>155 ± 42</td>
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<tr>
<td>Ku70−/− (n = 3)</td>
<td>2.98 ± 0.91</td>
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<tr>
<td>Ku80−/− (n = 2)</td>
<td>1.0 ± 0.5</td>
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<tr>
<td>Bone marrow</td>
<td></td>
</tr>
<tr>
<td>wild type (n = 4)</td>
<td>11.9 ± 3.3</td>
</tr>
<tr>
<td>Ku70−/− (n = 3)</td>
<td>7.2 ± 2.9</td>
</tr>
<tr>
<td>Ku80−/− (n = 2)</td>
<td>9.0 ± 3.0</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
</tr>
<tr>
<td>wild type (n = 4)</td>
<td>53 ± 20</td>
</tr>
<tr>
<td>Ku70−/− (n = 3)</td>
<td>6.5 ± 1.3</td>
</tr>
<tr>
<td>Ku80−/− (n = 2)</td>
<td>1.2 ± 0.5</td>
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</tbody>
</table>

Data shown are arithmetic means ± standard deviations from two to four individuals of each genotype analyzed at 4-6 wk of age. Cell numbers are shown per femur for bone marrow, and per whole organ for spleen and thymus.
which is similar to lin D-JH and V-DJ H rearrangements and DNA from wild-type littermate mice have only approximately 5-fold the fraction of B lineage cells in the mutant sample, since the thymus was amplified with primers that detected V-DJ rearrangements (26, 27); IVS, nonrecombining segment of the Ig locus between JH and C H1 (26). Multiple lanes (lanes 2, 4, 5, 6, 7, 8) were examined (not shown). The same thymus DNA samples were examined for Vj8–1,2 and Dd2 to J61 rearrangements (20, 27, 28). 100 ng DNA was used for Ku70+ (lanes 2 and 7), Ku80−/− (lane 1), and Ku70−/− mice (lane 7), and 1, 10, and 100 ng for wild-type mice (lanes 4–6). Controls include a 1-kb germline interval amplified in the Dd2 to J61 intervening region (germline), and a nonrecombining segment of the Ig locus between Jh and Cc1 (not shown). The same thymus DNA samples were examined for Vj8–1,2, and Dd2 to J61 recombination. Abbreviations: Dd2 to J61 rearrangements Vj8–1,2 and Vj8–1,2, V7183 and V558L to JH rearrangements (26); Vj8–1,2 to Vj8–1,2 rearrangements (28); germ-line, unrecombined DNA from the Dd2 to J61 interval; Dd2, Dd2 to J61 rearrangements (20, 27); IVS, nonrecombining segment of the Ig locus between Jh and Cc1 (26). Multiple lanes underneath each genotype label (Ku70−/−, Ku80−/−, and SCID) represent different individual animals. The rate and extent of rejoining of x-ray-induced DNA DSB in Ku70−/−, Ku80−/−, and Ku70−/−/+ cells were measured using AFIGE (31). Fibroblasts derived from day 13.5 embryos were exposed to 40 Gy of x-rays and returned to 37°C for repair. At various times thereafter, cells were prepared for AFIGE to quantitate DNA DSB (Fig. 5 B, top). DNA DSB were nearly completely rejoined in wild-type cells within ~2 h after radiation exposure. However, fibroblasts derived from Ku70−/− mice showed a drastically reduced ability to rejoin DNA DSB. A similar deficiency in DNA DSB rejoining was also observed in fibroblasts derived from Ku80−/− embryos. Despite the large differences observed in rejoining of DNA DSB between wild-type fibroblasts and fibroblasts derived from Ku70−/− or Ku80−/− mouse embryos, dose-response experiments showed that Ku70−/−, Ku80−/−, and wild-type fibroblasts were equally susceptible to x-ray-induced damage (Fig. 5 B, bottom). Thus, Ku deficiency primarily affects the ability of cells to rejoin radiation-induced DNA DSB without significantly affecting the induction of DNA damage.

**Discussion**

Absence of Ku70 results in radiation hypersensitivity and proportional dwarfism, as well as deficiencies in DNA DSB repair and TCR V(D)J recombination. Thus, Ku70−/− mice resemble Ku80−/− mice in several respects, but the two mutations differ in their effects on T and B cell development. Lack of Ku70 was compatible with TCR gene rearrangement and development of mature CD4+CD8+ and CD4−CD8+ T cells, whereas mature T cells were absent in Ku80−/− mice. In contrast, B cells failed to complete anti-
gen receptor gene rearrangement and did not mature in either Ku70−/− or Ku80−/− mice.

What could account for the differences we find in TCR and immunoglobulin gene rearrangements in the Ku70−/− mice? One implication of our findings is that there are alternative Ku70-independent rescue pathways that are compatible with completion of V(D)J recombination in T cells. It is likely at the critical phase of T cell maturation, other DNA repair activity may be stimulated (33, 34) and can functionally complement the Ku70 gene in T cell–specific V(D)J recombination. Since Ku80−/− mice are deficient in both T and B lymphocyte development, it is plausible that these yet to be identified alternative DNA repair pathways include Ku80. The much reduced level of Ku80 protein in Ku70−/− cells may in part account for the hypocellularity of Ku70−/− thymuses.

Although the role of Ku in V(D)J recombination is not molecularly defined, Ku has been proposed to protect DNA ends from degradation (18, 35), to activate DNA-PK (10, 11), and to dissociate the recombination-activating protein RAG–DNA complex to facilitate the joining reaction (20). These functions are not mutually exclusive, and they are all dependent on the interaction of Ku with DNA. Thus, the finding that Ku70 is not required for TCR gene rearrangement is particularly unexpected because the Ku70 subunit is believed to be the DNA-binding subunit of the Ku complex (36), and DNA-end–binding activity was not detected in Ku70-deficient cells (Fig. 1D).

In summary, our studies provide direct evidence supporting the involvement of Ku70 in the repair of DNA DSB and V(D)J recombination and the presence of a Ku70-independent rescue pathway(s) in TCR V(D)J rearrangement. The distinct phenotype of Ku70−/− mice should make them valuable tools for unraveling the mechanisms of DNA repair and recombination.
References


