depleted + 4 ng recombinant Ku (α-Ku+rKu) human (HeLa) cell extracts, or 10 μg parental (K1), Ku deficient (xrs6), or complemented (xrs6+Ku80) rodent (CHO) cell extracts. a, b, Activity assays were performed in triplicate, stopped after 5, 10, 20, and 40 minutes, and products analyzed by gel electrophoresis. Velocities were determined by quantification of the time course and linear regression. Velocities of extracts with Ku were compared to extracts without Ku by two tailed t-test; p<.01, **; p<.0001, **). c, Adduct formation was performed as in Figure 2b, c. Top panel is a total protein stain of the noted extracts, while the bottom panel is a phosphorimage of the corresponding protein-DNA adducts.

Supplemental Methods

Constructs and Protein Preparations. Recombinant purified human Ku, XRCC4-LigaseIV, Pol β and pol λ were expressed and purified as previously described28. The Ku 70 3A mutant involved substitution for alanine of K31, K160, and K164 (human cDNA) or K29, K158, and K162 (mouse cDNA) by the quickchange method (Stratagene). For XLF, a cDNA (the gift of K. Meek, MSU) was introduced into pFASTBAC1 with a C-terminal hexahistidine tag and purified by successive chromatography on HisTrap and Mono Q columns (GE Biosciences). DNA-PKcs was purified from HeLa cells as described in29. For whole cell extracts, HeLa and CHO cells were extracted in 10 mM TRIS-HCl pH 8.0, 600 mM KCl, 0.1% NP-40 substitute (Fluka), 20% glycerol, 1 mM EDTA, 1mM DTT, and protease inhibitors (Sigma, P8849). DNA was removed from extracts by precipitation with 0.1% polyethylene imine (Sigma), followed by sequential adsorption of the supernatant to phosphocellulose (Sigma), and hydroxyapatite (Biorad). Extracts were then dialysed or
diluted until equivalent to 10 mM TRIS pH 8.0, 250 mM KCl, 0.1% NP40, 10% glycerol, and 1 mM EDTA. HeLa cell extracts were further immunodepleted by two sequential adsorptions to protein A Sepharose beads pre-loaded with either pre-immune serum (Mock depleted) or serum from rabbits immunized against human Ku (Ku-depleted). HeLa and CHO cell extracts were then analyzed by Western blotting using antibodies against Ku (raised against purified human Ku heterodimer), pol β (ab26343, Abcam), actin (A2066, Sigma), and DNA-PKcs (Ab4, Neomarkers). In Supplemental Figure 7a comparison of the recombinant Ku standard (rKu; 5 ng) to Ku in mock depleted extracts (1.5 μg) allows for the estimation of the concentration of Ku in HeLa cell extracts as 4 ng Ku/μg extract.

DNA substrates. NHEJ substrates were generated with varied end structures as previously described. Briefly, substrates with 2, 3, or 4 nucleotide overhangs were created using polymerase chain reaction (PCR) to append the restriction sites Smul, BspQI, or Bsal (respectively) to either a 250 bp core sequence from the mouse Jk1 germline (used in Figure 1b) or a 300 bp variant modified to allow development of qPCR primers (used in all subsequent Figures). The resulting PCR products were cloned (TOPO-TA, Invitrogen) to generate plasmid templates used for further amplification. Amplified DNA fragments were then digested with appropriate restriction enzymes, and resulting substrate purified using a Qiaquick PCR clean up kit (Qiagen). Amplifications included $^{32}$P-α-dATP when substrates were used for in vitro reactions. Oligonucleotides for substrates were obtained from Integrated DNA Technologies, labelled at the 3’ end with $^{32}$P-cordycepin (Applied Biosystems), and annealed to complementary strands to form duplex substrates. The labeled strands were 5’Phos-UGGAAATCAAACGTAAGTAG for 5’dRP-DSB and
5’dRP-SSB, and 5’Phos-GUGGAAATCAACGTAAGTAGAATCCAAAGTCTCTTTTCTTCCG for AP-DSB. The appropriate labelled strands for 5’dRP-DSB, 5’dRP-SSB, and AP-DSB were annealed to 5’biotin-TEG-TCTACTTACGTTTGGATTTC, 5’biotin-TEG-TCTACTTACGTGATTTC, and 5’biotin-TEG-TGGAGGCAACCAAGC, and finally 5’biotin-TEG-TCGGAAGAAAGAGACTTTGGATTTC, respectively. A variant AP-DSB with the biotin terminal 22 bp deleted was employed in experiments described in Supplemental Figure 6a. All biotinylated substrate ends were blocked by pre-incubation of the substrate with 1 µM streptavidin (Pierce) for 5 minutes. The tetrahydrofuran containing substrate was generated by substituting dU in the labelled strand in AP-DSB with tetrahydrofuran (“dSpacer”; Integrated DNA Technologies). Bleocin damaged substrates were generated by annealing the oligonucleotide 5’TCTACTTACGTTTGGATTTCAGCTTGGTGCCCTCA to 5’TGGAGGCAACCAAGCTGGAAATCAAACGTAAGTAG, and incubating 100 fmol of the resulting duplex with 100 pmol Bleocin (EMD Biosciences) for 10 minutes at 37°C. For all other substrates, abasic sites were made by inclusion of dU at the appropriate site and incubation with 0.02 units uracil DNA glycosylase (NEB) per fmol substrate for 5 minutes at 37°C. Reduced AP site substrates were made by treating these glycosylated substrates with 50 mM NaBH₄ for 20 minutes on ice before an additional purification (Qiaquick PCR cleanup, Qiagen). The concentrations of all substrates were determined by Qubit.
(Invitrogen) using high sensitivity dsDNA (EJ substrates) or ssDNA (oligonucleotide substrates) stains.

**In vitro NHEJ assays.** 5 nM of 250 bp radiolabeled substrates with noted end structures were pre-incubated with 20 nM recombinant Ku, 10 nM DNA-PKcs, 40 nM XRCC4-LigaseIV, and 80 nM XLF in a standard reaction buffer (25 mM NaPO$_4$ pH 7.4, 125 mM KCl, 0.1 mM EDTA, and 1 mM DTT) supplemented with 10% polyethylene glycol for 5 minutes at 25°C. Reactions were started by addition of 2 mM MgCl$_2$ and 100 µM ATP and incubated at 37°C, stopped by deproteinization, and analyzed by native 5% polyacrylamide gel electrophoresis (PAGE).

**Cellular NHEJ assays.** Dermal fibroblasts from Ku70$^{-/-}$ p53$^{-/-}$ mice were infected with retroviruses with a wild type mouse Ku70 cDNA, the mouse Ku70 3A mutant, or empty vector (pBABE-puro) using standard techniques and selected for at least 4 days using 2 µg/ml puromycin (this dose and time was sufficient to fully kill uninfected cultures treated in parallel). 1x10$^6$ puromycin resistant cells were then transfected with 5 ng substrate and 1.5 µg pMAX-GFP tracer using the Amaxa nucleofector II, a MEF-2 kit, and program A-023 (Lonza). Transfected cells were harvested 5 hr later and small molecular weight DNA recovered in a Hirt supernatant$^{31}$. Joining was evaluated both by semi-quantitative and real time PCRs (qPCR).

Semi-quantitative PCRs (30 cycles) were performed with 2.5 µCi $^{32}$P-α-dATP (without dye). Products were analyzed after digestion as appropriate (to characterize junctions) and electrophoresis on 8% polyacrylamide gels under native conditions.

qPCRs were performed with Power-SYBR mix and an ABI 7300 (Applied Biosystems) to
quantify both substrate and head-to-tail junctions. Levels of head-to-tail junctions were adjusted according to the results of substrate qPCR to account for differences in transfection and sample recovery (though substrate-specific qPCRs rarely varied by more than 30% from the mean for a given experiment).

All joining efficiencies comparing different substrates were assessed in parallel, using different aliquots of the same cells, for each transfection. In Figure 1d and Supplemental Figure 3 we first determined the estimated maximum efficiency (100%) of joining as the average threshold cycle ($c_t$) determined for all experiments performed with undamaged ends (5’AT, 5’ATAT) transfected into Ku70 complemented cells. Joining for each individual experiment was then compared to this estimated maximum (“joining efficiency”).

Expression levels of wild type and Ku 70 3A could not be accurately assessed by western analysis for technical reasons. We therefore used undamaged control substrate to ensure Ku 70 3A complemented cells were not generally defective (e.g. due to insufficient complementation, for whatever reason). Highly efficient and consistent complementation was further validated by also assessing joining efficiencies with undamaged substrate in control Ku70-deficient cells (infected with empty vector) in these experiments. Relative to cells complemented with wild type Ku70, Ku-deficient cells were only 5.4% +/- 2.2 (mean for the 4 transfections, +/- s.e.m.) as active, or only 6.0% +/- 2.5 as active relative to cells complemented with Ku 70 3A. These experiments were repeated twice for each of two different virus preparations/infections (complementations). In agreement with consistent complementation, independent viral preps/infections did not contribute significantly to the overall experimental variation observed in results with either wt or Ku70 3A construct.
Two-tailed t-tests were used for tests of statistical significance (Prism, Graphpad).

Supplemental references

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