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Ku is a 5'dRP/AP lyase that excises nucleotide damage near broken ends

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Abstract

Mammalian cells require Nonhomologous end joining (NHEJ) for efficient repair of chromosomal DNA double-strand breaks[1]. A key feature of biological sources of strand breaks is associated nucleotide damage, including base loss (abasic or AP sites)[2]. At single strand breaks, 5' terminal abasic sites are excised by pol β's 5'dRP lyase activity[3],[4],[5],[6]: we show here *in vitro* and in cells that accurate and efficient repair by NHEJ of double-strand breaks with such damage similarly requires 5'dRP/AP lyase activity (Figure 1a). Classically defined NHEJ is moreover uniquely effective at coupling this end-cleaning step to joining in cells, helping distinguish this pathway from otherwise robust alternate NHEJ pathways. Surprisingly, the NHEJ factor Ku can be identified as an effective 5'dRP/AP lyase. Similar to other lyases[7], Ku nicks DNA 3' of an abasic site by a mechanism involving a Schiff base covalent intermediate with the abasic site. We demonstrate using cell extracts that Ku is essential for efficient removal of AP sites near double-strand breaks and, consistent with this result, joining of such breaks is specifically reduced in cells complemented with a lyase-attenuated Ku mutant. Ku had previously been presumed only to recognize ends and recruit other factors that processed ends; our data supports an unexpected direct role for Ku in end processing steps as well.

Abasic sites are frequently associated with double-strand breaks generated by ionizing radiation, treatment with radiomimetic drugs[2],[8], after aborted base excision repair[9],[10], or as an intermediate in class switch recombination[11],[12]. We first addressed how NHEJ resolves such damage *in vitro*, using 250 bp DNA substrates with abasic sites located near their 5'

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Author Contributions Experiments were designed by S.A.R. and D.A.R. *In vitro* experiments were performed by S.A.R, N.S, M.D.B, and D.A.R. Mutagenesis and protein purification were performed by S.A.R. and D.A.R. S.A.R. C.S., J.M.H., and M.D.B. performed cellular experiments. P.H. provided Ku70 knockout dermal fibroblasts. S.A.R. and D.A.R. wrote the manuscript with the aid of N.S. and M.D.B.

Supplemental Information.

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termini (5'dRP-EJ, AP-EJ, Figure 1b) and purified human NHEJ core factors (Ku, DNA-PKcs, XLF/Cernunnos, and XRCC4-LigaseIV). Joining was unexpectedly efficient without addition of a known 5'dRP/AP lyase (Figure 1b, lanes 2 and 5). Nevertheless, characterization of junctions indicated ligation occurred after precise excision of the abasic site (Supplemental Figure 1c), and ligation was negligible if 5'dRP/AP lyase activity was blocked[3] by prior reduction of substrate abasic sites (Figure 1b, "R"). NHEJ must therefore employ lyase activity for excision of 5'terminal abasic sites in a manner similar to short patch base excision repair (BER) (Figure 1a). Additionally, our purified NHEJ core factor preparations are surprisingly sufficient to perform this function.

We next asked if a 5'dRP/AP lyase was also important in NHEJ of such substrates in cells. We used Ku70 and p53 deficient mouse dermal fibroblasts[13] either complemented by expression of a mouse Ku70 cDNA (+Ku70) or an empty vector control (+vector), and introduced into these cells variations of the abasic site-containing substrates described above as well as undamaged control versions of these substrates. Quantitative polymerase chain reactions (qPCR) were then used to assess efficiency of overall recovery as well as head-to-tail joining (substrate and junction qPCRs; Figure 1c).

Cells proficient in classically-defined NHEJ (+Ku70) were equally effective in joining substrates with an embedded normal AP site (5'A○AT) as they were in joining undamaged control substrates (5'ATAT, 5'AT) (Figure 1d; columns 1, 3 and 7). Using restriction enzyme digestions diagnostic for specific junctions (Supplemental Figure 2a) we further determined undamaged ends (5'ATAT, 5'AT) were usually joined without deletions or additions (Supplemental Figure 2b), as expected. By comparison, the 5'A○AT substrate was typically joined after precise excision of the AP site, but with no further addition or deletion (Supplemental Figure 2b), consistent with *in vitro* data and the model in Figure 1a. Also consistent with *in vitro* data, reduction of the AP site (and consequent blocking of 5'dRP/AP lyase activity; 5'A●AT) decreased cellular ligation ~20 fold (Figure 1d, columns 3 and 5). The majority of junctions that were formed contained deletion of DNA flanking the reduced AP site (Supplemental Figure 2b, c). Excision of AP sites near DSBs by a 5'dRP/AP lyase is thus critical for efficient and accurate resolution of such ends by cellular NHEJ (Figure 1a).

Consistent with other studies[14,15] (reviewed in[16]) an alternative to NHEJ (Alt-NHEJ) allows for significant joining of undamaged ends in Ku70 deficient cells (Figure 1d, columns 1 and 2, 7 and 8). In comparison to classical NHEJ however, a near-terminal abasic site is a clear barrier to Alt-NHEJ and reduction of the AP site no longer has a significant impact (Figure 1d, columns 2, 4, 6). Even the intrinsically less stable terminal abasic site (5'dRP) is a strong barrier to Alt-NHEJ, but not Ku-dependent NHEJ (Supplemental Figure 3). We conclude Ku-dependent NHEJ is uniquely able to couple 5'dRP/AP lyase activity to joining (Figure 1a), and that this activity is critical for efficient and accurate resolution of ends with such damage.

The experiment described in Figure 1b indicated that one of the known NHEJ core factors might be a 5'dRP/AP lyase. Of the four core factors employed in this assay only Ku had significant activity on its own: Ku excised both terminal (5'dRP, Supplemental Figure 4a) and penultimate (AP sites, Figure 2a) abasic sites, resulting in species that co-migrate with

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alkali-cleaved controls (Figure 2a, lane 8). This is consistent with cleavage 3' of the abasic site and production of a 5' phosphorylated terminus. Activity was also blocked by substitution of abasic sites with an analogue resistant to lyase activity[3] (Figure 2a, lane 11). 5'dRP/AP lyases can be further characterized by trapping of a covalent reaction intermediate. Nucleophylic lysines in lyase active sites form a Schiff base with the 1′ carbon of the abasic site (Figure 2b), and this normally triggers cleavage of the phosphodiester bond 3' of the abasic site through β elimination[7]. Addition of $NaBH_4$ reduces this intermediate to instead make a stable protein-DNA adduct - if the DNA substrate is radioactive, $NaBH_4$ treatment radiolabels the active lyase. As expected, adducted species required both the abasic site and $NaBH_4$ and were consistent with DNA adducted to Ku70 and Ku80 (confirmed by mutant analysis, Figure 2c). Importantly, $NaBH_4$ trapping identifies even weak nucleophiles. In this regard DNA-PKcs, though also capable of forming an adduct (Supplemental Figure 4b), typically does little to directly impact activity of the Ku heterodimer (e.g. Figure 2a) whether DNA-PKcs is active as a kinase or not (Supplemental Figure 4c).

We further characterized Ku70's contribution to activity by systematic mutagenesis of 21 candidate catalytic lysines. Mutation of K31 alone diminished Ku70's ability to form a Schiff base (unpublished data), but additional mutation of two nearby[17] lysines (K160, K164; Supplemental Figure 5a) was required to completely ablate adduct formation (Ku70 3A, Figure 2c). However, the Ku 70 3A mutant only reduces 5'dRP/AP lyase activity 2 fold (Supplemental Figure 5c) and promotes adduction with Ku80 (Figure 2c), indicating nucleophiles within Ku80 can at least partly compensate. Similar observations of compensating activity have been observed after mutation of the primary nucleophiles in other biologically important 5'dRP lyases (e.g. Pol4 *KK247*: *:248AA*[18], Pol β K72A[19]). The modest lyase defect observed with the Ku70 3A mutant *in vitro* was nevertheless sufficient to cause a comparable defect in NHEJ of 5'dRP containing substrates in cells (Figure 2d, Supplemental Figure 5d). Importantly, the mutant heterodimer is not significantly defective for DNA end binding *in vitro* (Supplemental Figure 5b) or joining of the undamaged control substrate in cells (Figure 2d, Supplemental Figure 5d). Notably, general loss of function was observed with a more severe perturbation of the candidate active site (deletion of Ku70 aa 4–34; unpublished data). Taken together, this data is consistent with a significant and non-redundant role for Ku's 5'dRP/AP lyase activity in NHEJ of ends with associated abasic sites.

Is Ku a robust 5'dRP/AP lyase? 5'dRP and AP sites are excised by Ku with half lives of 2.7 and 7.0 minutes, respectively (Supplemental Figure 6a). These rates are readily accommodated within the typical half life of radiation-induced double-strand breaks in cells[20] and are in the range of rates of dRP removal as performed during BER/SSBR[4,21,22]. However, proximity of an abasic site to a DSB can present a barrier for canonical dRP/AP lyases. For example, while Pol β is proficient at this step at single strand breaks (5'dRP-SSB) (where Ku is not significantly active)[23] (also Supplemental Figure 6b), its activity is greatly reduced at DSBs. Ku, however, is active at DSB ends - approximately 10 fold more active than is pol β (Supplemental Figure 6b). Another pol X member, pol λ, is implicated in NHEJ, but its 5'dRP lyase activity[24] is even more restricted to single strand breaks than pol β

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(Supplemental Figure 6c). These results are consistent with genetic studies arguing against an important role for lyase activity of *pol 4,* the only pol X member in *S. cerevisiae*, in cellular NHEJ of ends with nearby abasic sites[25].

We next tested whole cell extracts to more comprehensively assess the importance of Ku's 5'dRP/AP lyase activity. Strikingly, extracts specifically deficient in Ku (Supplemental Figure 7a) were 70 fold (human cell extracts) or 5 fold (rodent cell extracts) less active than matched control extracts in excising DSB terminal abasic sites (Figure 3a and b). Moreover, human cell extracts have much higher specific activity than rodent cell extracts, consistent with Ku's greater abundance in primates[26]. Ku is also the primary factor in extracts that forms covalent protein-DNA intermediates trapped by $NaBH_4$ (Figure 3c), arguing that requirement for Ku in activity assays is not due to recruitment by Ku of other 5'dRP/AP lyases. Similar results were observed using DNA damaged *in vitro* with the radiomimetic drug bleocin (Supplemental Figure 7b), consistent with an important role for Ku in cleaning termini with abasic site damage derived directly by oxidation[8] as well as after glycolysis (Figures 1–3). We therefore conclude that Ku easily accounts for the majority of 5'dRP/AP lyase activity in cell extracts when AP sites are near 5' termini of double-strand breaks.

We show both *in vitro* and in cells that NHEJ can and must “clean” termini of abasic sites, a class of nucleotide damage commonly associated with strand breaks, before such broken ends can be joined. Notably, abasic sites are a strong barrier to Alt-NHEJ, suggesting a general inability of Alt-NHEJ to couple end cleaning steps to joining might help explain why mammalian cells deficient in classical NHEJ are so radiosensitive[1,27]. That NHEJ utilizes a 5'dRP/AP lyase for excision of near terminal abasic sites is a striking parallel to short patch BER/SSBR (Figure 1a), and has clear advantages: the ability to specifically excise damage allows for more conservative resolutions than less directed end processing mechanisms. We further surprisingly identify the “scaffolding” factor Ku as the 5'dRP/AP lyase NHEJ employs for this function.

Methods Summary

For *in vitro* end joining and lyase reactions recombinant purified Ku, XRCC4-LigaseIV, XLF/Cernunnos, and/or DNA-PKcs purified from Hela cells were incubated with radiolabeled substrates before analysis by electrophoresis. For cellular NHEJ assays a dermal fibroblast line from a mouse deficient in Ku70 and p53[13] was engineered by retroviral transduction to express wild type mouse Ku70, Ku70 3A, or an empty vector control. Expressing cells were then purified by selection for puromycin resistance. NHEJ substrates were introduced into these cells by electroporation, harvested, and sample recovery and NHEJ efficiency assessed by qPCR. NHEJ accuracy was further assessed by digestion of amplified junctions with a restriction enzyme diagnostic for predicted products.

Supplementary Material

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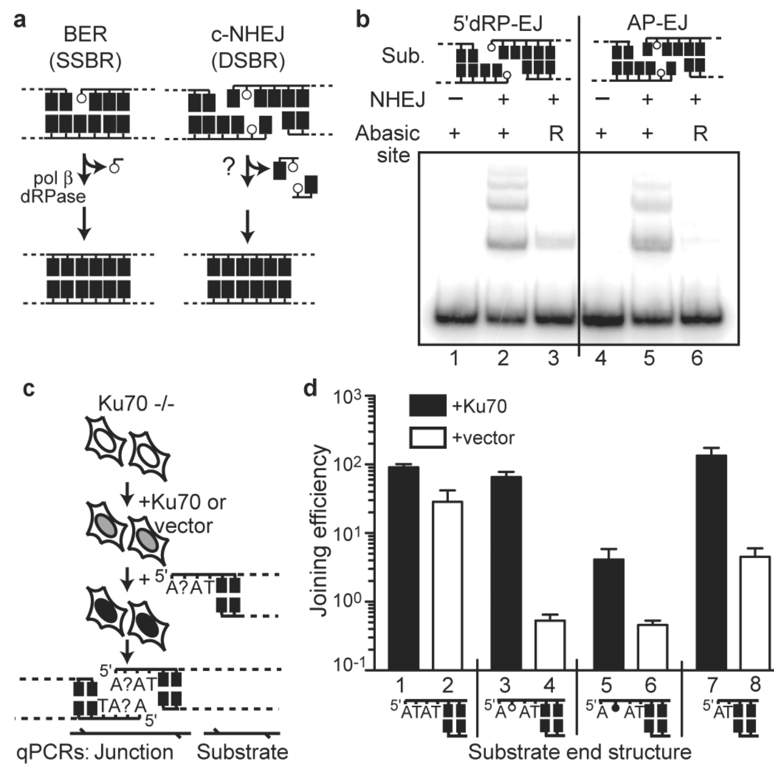

Figure 1.

NHEJ of ends with abasic sites. Substrate cartoons show position of abasic site (open circle; if reduced, closed circle) relative to intact nucleotides (blocks/letters). **a**, Repair of strand breaks (single strand, SSB; double-strand, DSB) with associated abasic sites. **b**, Radiolabeled 250 bp substrates were incubated with purified Ku, DNA-PKcs, XRCC4-LigaseIV, and XLF at 37°C for 20 minutes (5'dRP-EJ) or 60 minutes (AP-EJ) and products detected by electrophoresis and phosphorimaging. R; abasic sites reduced. **c** and **d**, Substrates with end structures varied as noted in cartoons were introduced into Ku70$^{-/-}$ fibroblasts complemented with Ku70 or empty vector standard. Recovered DNA was then assessed for efficiencies of substrate recovery and joining by qPCRs using substrate and junction primer pairs, respectively. Error bars reflect the standard error of the mean (s.e.m.) from 4 independent transfections.

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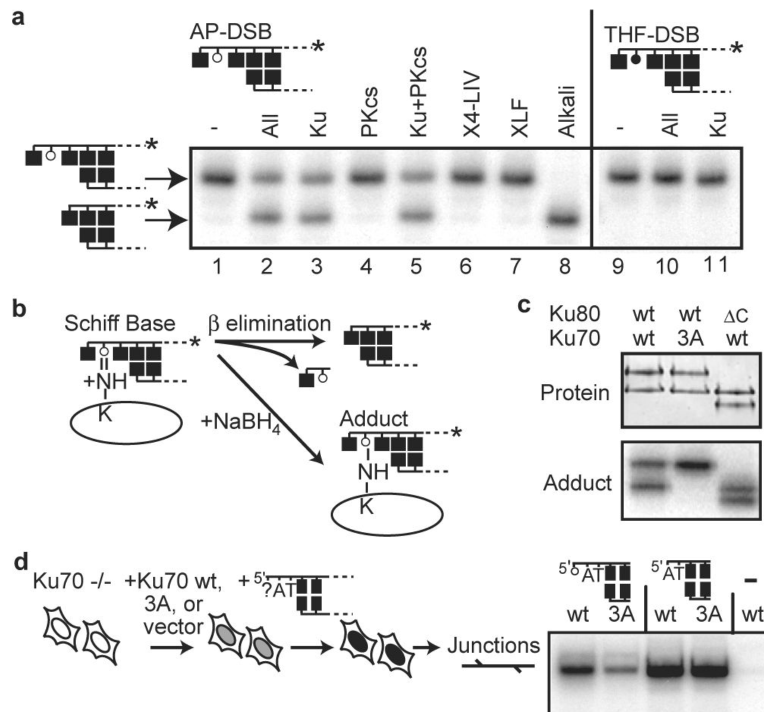

Figure 2.

5'dRP/AP lyase activity of purified NHEJ factors. **a**, 1 nM radiolabeled 40 bp AP-DSB substrate with abasic site (open circle, lanes 1–8) or lyase-resistant analogue (tetrahydrofuran; filled circle, lanes 9–11) were incubated with 2.5 nM purified proteins as indicated at 37°C for 30 minutes and reactions analysed by electrophoresis. **b**, Addition of $NaBH_4$ at the start of the reaction reduces the Schiff base covalent intermediate, forming a stable adduct between an abasic site within a radiolabeled DNA substrate and the 5'dPR/AP lyase. **c**, Adducts were formed as described in b with purified wild type heterodimer (wt), a heterodimer of Ku70 3A (Ku70 with K31A, K160A, and K164A substitutions) and wild type Ku80, and a heterodimer of wild type Ku70 and Ku80 lacking the C terminal 162 amino acids (C). Top panel shows purified heterodimers, while bottom panel shows phosphorimage of corresponding protein-DNA adducts. **d**, Ku70–/– fibroblasts were complemented with wild type Ku70, Ku70 3A, or empty vector, and then transfected with various substrates as indicated. Joining was evaluated by semi-quantitative PCR (shown for a pool of samples from 4 independent transfections) or qPCR (Supplemental Fig. 5d). Consistent complementation was validated using the undamaged control substrate (5'AT); see also methods.

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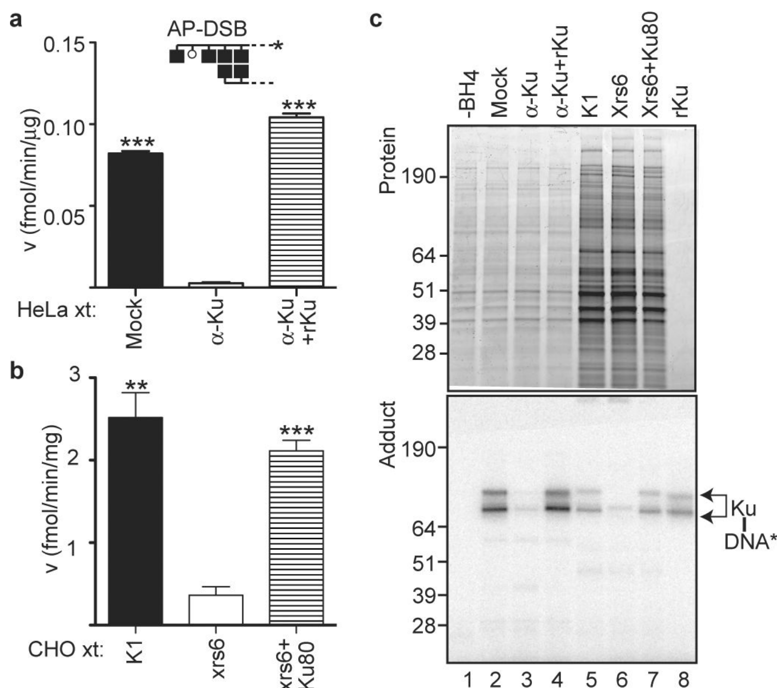

Figure 3.

AP lyase activity of cell extracts with or without Ku. **a, b, c,** 1 nM AP-DSB at 37°C was incubated with 1 μg Mock depleted (Mock), Ku depleted (α-Ku), or Ku 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.