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The Ku80 Carboxy Terminus Stimulates Joining and Artemis-Mediated Processing of DNA Ends[∇]

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Repair of DNA double-strand breaks (DSBs) is predominantly mediated by nonhomologous end joining (NHEJ) in mammalian cells. NHEJ requires binding of the Ku70-Ku80 heterodimer (Ku70/80) to the DNA ends and subsequent recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PK$_{CS}$) and the XRCC4/ligase IV complex. Activation of the DNA-PK$_{CS}$ serine/threonine kinase requires an interaction with Ku70/80 and is essential for NHEJ-mediated DSB repair. In contrast to previous models, we found that the carboxy terminus of Ku80 is not absolutely required for the recruitment and activation of DNA-PK$_{CS}$ at DSBs, although cells that harbored a carboxy-terminal deletion in the Ku80 gene were sensitive to ionizing radiation and showed reduced end-joining capacity. More detailed analysis of this repair defect showed that DNA-PK$_{CS}$ autophosphorylation at Thr2647 was diminished, while Ser2056 was phosphorylated to normal levels. This resulted in severely reduced levels of Artemis nuclease activity in vivo and in vitro. We therefore conclude that the Ku80 carboxy terminus is important to support DNA-PK$_{CS}$ autophosphorylation at specific sites, which facilitates DNA end processing by the Artemis endonuclease and the subsequent joining reaction.

DNA double-strand breaks (DSBs) classify among the most detrimental DNA damages, because they have the ability to cause chromosome breakage and translocations. DSBs are readily caused by common exogenous and endogenous agents, including certain oxygen radicals, products of normal metabolism, and ionizing radiation. Effective genomic maintenance therefore requires the presence of a mechanism to repair DSBs. DSB repair in eukaryotic cells is executed by either homologous recombination or by nonhomologous end joining (NHEJ) (15, 30).

In vertebrates, DSB repair is not only essential for genomic maintenance, but also for the development of a working immune system. The assembly of immunoglobulin or T-cell receptor genes via V(D)J recombination routinely necessitates the introduction and subsequent NHEJ-mediated repair of DSBs (13).

The NHEJ pathway facilitates DSB repair by direct ligation of the two ends of a broken DNA molecule (31, 36). This requires the sequential loading of several enzymes on both DNA ends. The first event in NHEJ-mediated repair is the association of a Ku70-Ku80 heterodimer (Ku70/80) with each DNA terminus. The Ku70/80 molecule has a ring-shaped structure, made up by the amino-terminal and central domains of both the Ku70 and the Ku80 polypeptides, which exactly fits a DNA helix in its center (33).

The DNA-Ku complex functions as a scaffold to attract the other known NHEJ factors to the DSB. One of the enzymes that are recruited to the DNA-Ku scaffold is the DNA-dependent protein kinase catalytic subunit (DNA-PK$_{CS}$), a 469-kDa serine/threonine kinase. The Ku-DNA-PK$_{CS}$ complex is commonly referred to as DNA-PK. It has been well established that the DNA-PK$_{CS}$ kinase activity is essential for efficient DSB repair, although the mechanism via which DNA-PK$_{CS}$ exerts its function is a matter of current debate (19, 35, 36). Several autophosphorylation sites have been mapped in the DNA-PK$_{CS}$ protein. The most important clusters are found between residues 2609 and 2647 (ABCDE cluster) and between residues 2023 and 2056 (PQR cluster). Phosphorylation of the ABCDE cluster was found to specifically stimulate processing and joining of DNA ends, while PQR phosphorylation reduced the level of DNA end processing (35). These findings prompted a model in which DNA-PK$_{CS}$ functions as a gatekeeper molecule that regulates access to the DNA termini by changing its phosphorylation status (35). Therefore, DNA-PK$_{CS}$ autophosphorylation may regulate the next steps in the NHEJ process.

These next steps include the processing and joining of DNA ends. Processing enzymes prepare nonligatable DNA termini, primarily blocked ends and incompatible single-strand overhangs, for subsequent ligation by the XRCC4/ligase IV complex. The chemistry of the ligation reaction necessitates the addition of 5′ phosphate groups or the removal of 3′ phosphate groups by polynucleotide kinase (3). Processing of single-strand overhangs is performed by either filling or resection and therefore requires a polymerase or a nuclease, respectively (16, 36). Several enzymes with single-strand filling capability, in-

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cluding polymerase λ, polymerase μ, and terminal deoxynucleotidyltransferase, have been suggested to function as processing enzymes during NHEJ (16). In contrast, only one nuclease has been conclusively shown to play a role in NHEJ: the endonuclease Artemis.

Artemis was first described as an essential contributor to V(D)J recombination, catalyzing the opening of hairpin structures at coding ends (17, 21, 24). However, because Artemis deficiency not only causes impairment of V(D)J recombination but also increased sensitivity to DSB-inducing ionizing radiation, it was soon recognized that Artemis may act as a processing enzyme for other types of DNA ends during NHEJ as well. The Artemis protein forms a complex with DNA-PK and carries the endonuclease activity that is necessary for the hairpin opening or overhang processing (14, 17). It is likely that the Artemis protein is recruited to the repair complex by interaction with the DNA-Ku–DNA-PK_{CS} complex.

Because the NHEJ core factors DNA-PK_{CS}, XRCC4/ligase IV, and Artemis are attracted to a DSB by the DNA-Ku scaffold, we set out to examine the influence of specific deletions of the Ku80 protein on the recruitment and activation of these core factors. It has been previously reported that the Ku80 carboxy terminus is important for effective NHEJ, evidenced by the fact that deletion of the Ku80 carboxy terminus results in markedly increased sensitivity to ionizing radiation and decreased retention of DNA-PK_{CS} at DNA ends (11). Several authors have suggested that the Ku80 carboxy terminus mediates activation of the DNA-PK_{CS} kinase and may therefore be directly responsible for regulation of the NHEJ process (11, 12, 25).

In contrast to that hypothesis, we here show that the Ku80 carboxy terminus is not an essential prerequisite for recruitment or activation of the DNA-PK_{CS} kinase in vivo. Surprisingly, however, deletion of the Ku80 carboxy terminus resulted in less efficient phosphorylation of specific DNA-PK_{CS} autophosphorylation sites and diminished Artemis endonuclease activity. These findings provide a comprehensive explanation for the increased radiation sensitivity that is associated with deletion of the Ku80 carboxy terminus.

MATERIALS AND METHODS

Cell culture. DNA-PK_{CS}-deficient V3 cells (38) and Ku80-deficient Xrs6 cells (7) were maintained in alpha-minimum Eagle medium with 10% fetal calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Stable cell lines expressing YFP-tagged DNA-PK_{CS} or YFP-tagged Ku80 were maintained with 400 μg of G418/ml. Throughout experimentation, the absence of endogenous Ku80 in Xrs6 cells was verified by Western blot analysis.

IR survival assay. Cell survival was measured by using a colony formation assay. Briefly, 300 cells per cell line were plated in triplicate in 60 mm dishes 2 to 6 h prior to ionizing radiation (IR). Dishes were irradiated at the indicated doses, followed by incubation for 10 to 12 days. Colonies (>50 cells) were visualized by staining with crystal violet solution (0.5% crystal violet, 1% formaldehyde in phosphate-buffered saline [PBS]) and subsequently counted.

Protein expression and purification. Ku70/80 and fluorescently tagged variants were expressed with a His_6 tag on the Ku70 moiety in a baculovirus expression system. They were purified as described, using Ni^{2+} Sepharose and DNA-cellulose columns (22). DNA-PK_{CS} was purified from HeLa cells essentially as described previously (2). The absence of Ku70/80 from this protein preparation was carefully checked by using Western blot analysis and DNA-PK activity assays. XRCC4/ligase IV was expressed in *Escherichia coli* and purified as described previously (20). Artemis-YFP-His_6-HA was expressed in a baculovirus expression system and purified on a Ni^{2+} Sepharose column. Protein was eluted in 20 mM HEPES-KOH (pH 7.5)–100 mM KCl–2 mM β-mercaptoethanol–300 mM imidazole. Artemis-containing fractions were directly loaded onto a Resource Q column (Pharmacia) and eluted with a linear gradient from 50 mM to 1 M KCl in 25 mM HEPES-KOH (pH 7.5)–1 mM dithiothreitol (DTT)–10% glycerol. Fractions containing Artemis-YFP-His_6-HA were collected. All proteins were fast frozen in liquid N_2 and stored at −80°C.

EMSA analysis. Electrophoretic mobility shift assay (EMSA) analysis was carried out by mixing the annealed product of radiolabeled DG182 (5′-ATCCCTTAAGGTGCAGATGAACTTCAGGGTCAG) and DG256 (5′-biotin-CTGACCCTGAAGTTCATCTGCACCTTAAGG) (0.2 pmol) with Ku70/80 (20 ng/μl), DNA-PK_{CS} (20 ng/μl), or XRCC4/ligase IV (20 ng/μl), as indicated, in a 10-μl reaction containing 100 mM KCl, 25 mM Tris-HCl (pH 8), and 1 mM DTT. After 20 min at room temperature, reaction mixtures were separated on 4% polyacrylamide gels in 0.5× Tris-borate-EDTA buffer, and products were visualized by phosphorimaging.

Laser microirradiation and FRAP measurements. Introduction of DSBs by microirradiation with a pulsed 365 nm nitrogen laser and fluorescence recovery after photobleaching (FRAP) analysis was performed as previously described (28). For FRAP analysis, images were collected every 30 s, and the fluorescence intensity of the photobleached region was measured. The intensities were normalized to the prebleach level (set to 1) and postbleach level (set to 0).

DNA-PK_{CS} kinase and autophosphorylation assay. DNA-PK_{CS} kinase assays were carried out by using the Signatect DNA-PK assay system (Promega) according to the manufacturer's specifications with 10 ng of purified DNA-PK_{CS} and increasing concentrations of Ku70/80 protein, as indicated. Autophosphorylation assays were performed as previously described (37) in a buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.08 mg of bovine serum albumin (BSA)/ml, and 25 μM ATP.

Immunofluorescence staining and antibodies. Immunofluorescence staining was performed as previously described (4). Briefly, microirradiated cells were fixed with cold methanol for 20 min on ice and permeabilized for 10 min in PBS containing 0.5% Triton X-100. After blocking with PBS containing 5% BSA for 20 min, the cells were incubated with the primary antibodies. Anti-pS2056 and anti-pT2647 polyclonal antibodies were generated as previously described (1). Anti-pT2609 was purchased from Abcam.

End-joining and V(D)J assay. End-joining and V(D)J recombination assays using pDVG94 and pDVG93, respectively, were carried out as previously described (32).

Artemis endonuclease assay. Oligonucleotide hairpin substrate was prepared by radiolabeling of DG262 (5′-GAACTTCAGGGTCAG), followed by annealing to DG261 (5′-biotin-CTGACCCTGAAGTTCATCTGCACCTTAAGGCCTTAAGGTGCAGAT) and ligation using T4 DNA ligase. Artemis nuclease assays were carried out in 10-μl reaction mixtures containing 10 mM Tris-HCl (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.5 μg of BSA, 100 ng of Ku70/80 (or a mutant form), 50 ng of DNA-PK_{CS}, 100 ng of Artemis-YFP-His_6-HA, and 0.2 pmol of hairpin substrate DG261/262. Reaction mixtures were incubated for 60 min at 37°C, and reaction products were separated on 8% polyacrylamide gels in TBE-urea. Cleavage products were visualized by phosphorimaging.

RESULTS

The Ku80 carboxy terminus is required for DSB repair but not for recruitment of Ku70/80 to DSBs. We first verified the notion that the Ku80 carboxy terminus plays an intricate role during DSB repair. Therefore, we complemented Ku80-deficient Xrs6 cells with wild-type Ku80 and with mutant forms of Ku80 from which either the complete carboxy terminus or the carboxy-terminal 14 amino acids were removed (Fig. 1A). The radiosensitivity of these stable cell lines was determined by performing a survival assay after exposure to gamma radiation. As reported previously (11), both deletion of the complete carboxy terminus and deletion of the terminal 14-amino-acid region resulted in significant sensitivity to ionizing radiation (Fig. 1B). However, cells complemented with the mutant versions of Ku80 were less radiosensitive than Ku80-deficient Xrs6 cells. We conclude that the Ku80 carboxy terminus strongly contributes to effective DSB repair, although the

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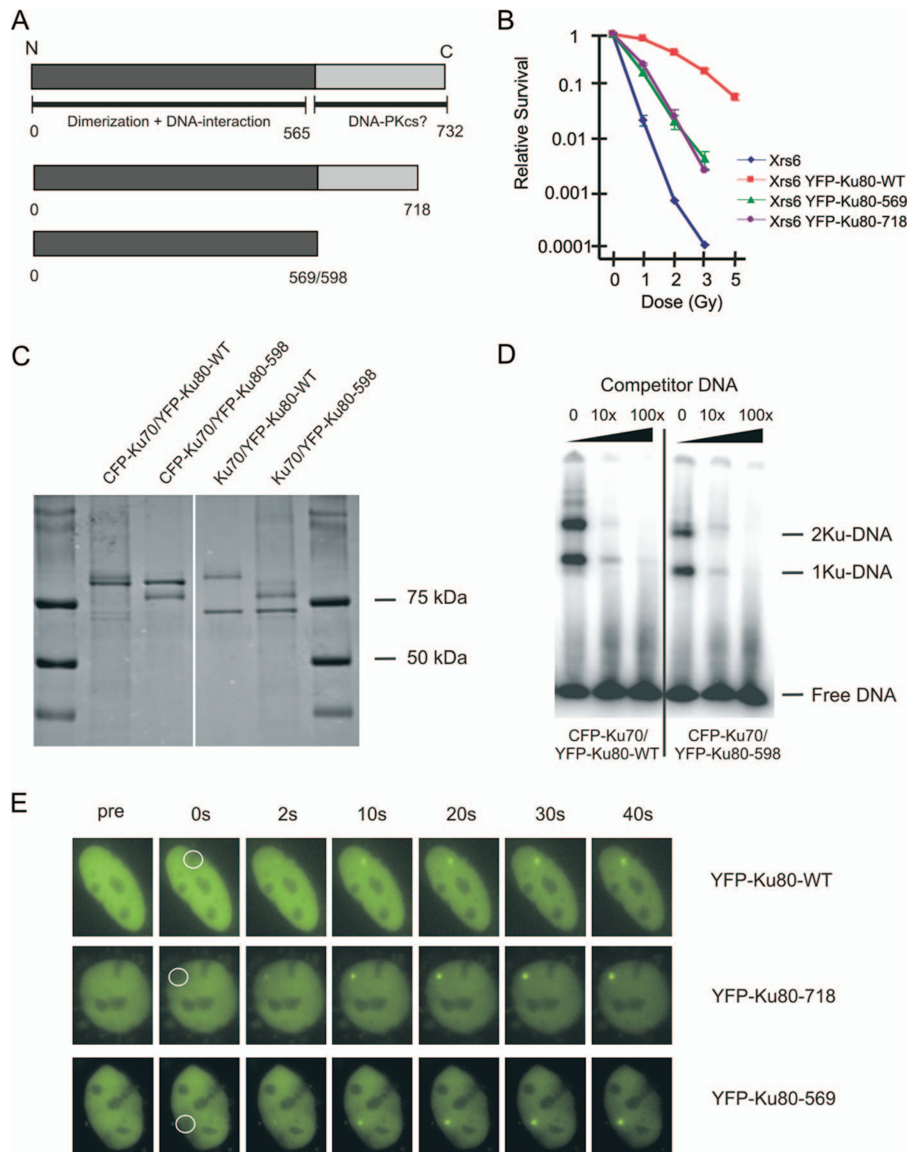

FIG. 1. The Ku80 carboxy terminus is important for DSB repair but not for recruitment of Ku70/80 to DSBs. (A) Schematic overview of the Ku80 deletion mutants used in the present study. (Top panel) The Ku80-WT (wild type) protein consists of 732 amino acids. (Middle panel) The Ku80-718 mutant lacks 14 carboxy-terminal amino acids. (Bottom panel) The Ku80-598 mutant lacks the entire carboxy terminus. In the present study we alternate between the use of a 598 and a 569 mutant. (B) IR survival of Xrs6 cells and (YFP-labeled) Ku80-WT-, Ku80-569-, or Ku80-718-complemented Xrs6 cells. (C) Purified Ku70/Ku80-WT and Ku70/Ku80-598 dimers. Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue. (D) EMSA analysis, demonstrating the capability of the Ku70/Ku80-WT and Ku70/Ku80-598 dimers to associate with DNA in vitro. With decreasing amounts of unlabeled competitor DNA, the relative amount of detectable Ku-DNA species increases. (E) Accumulation of (YFP-labeled) Ku80-WT, Ku80-569, and Ku80-718 at DSB sites created by 365-nm laser IR in vivo. The white circle at t = 0 indicates the irradiated spot.

amino-terminal 569 amino acids of Ku80 can partially rescue the repair defect of Xrs6 cells.

We subsequently studied the ability of the Ku80 carboxy-terminal deletion mutant to associate with Ku70 and to bind DNA ends in vitro. We therefore expressed either wild-type or mutant Ku80 protein in complex with wild-type Ku70 protein in a baculovirus expression system and purified these complexes to near homogeneity (Fig. 1C). The purified dimers were then incubated with a radioactively labeled DNA fragment. An EMSA demonstrated that both wild-type and deletion mutant dimers were able to bind to the DNA fragment and were equally sensitive to the addition of unlabeled competitor DNA (Fig. 1D), showing that the Ku80 carboxy terminus is not required for heterodimer formation or DNA end binding.

Next, we verified that Ku70/80 also binds to DSBs in vivo in the absence of the Ku80 carboxy terminus. We utilized a previously described method of laser microirradiation (28) to introduce a small region of DSBs in the nucleus of Xrs6 cells that were complemented with (YFP-labeled) wild-type or mutant

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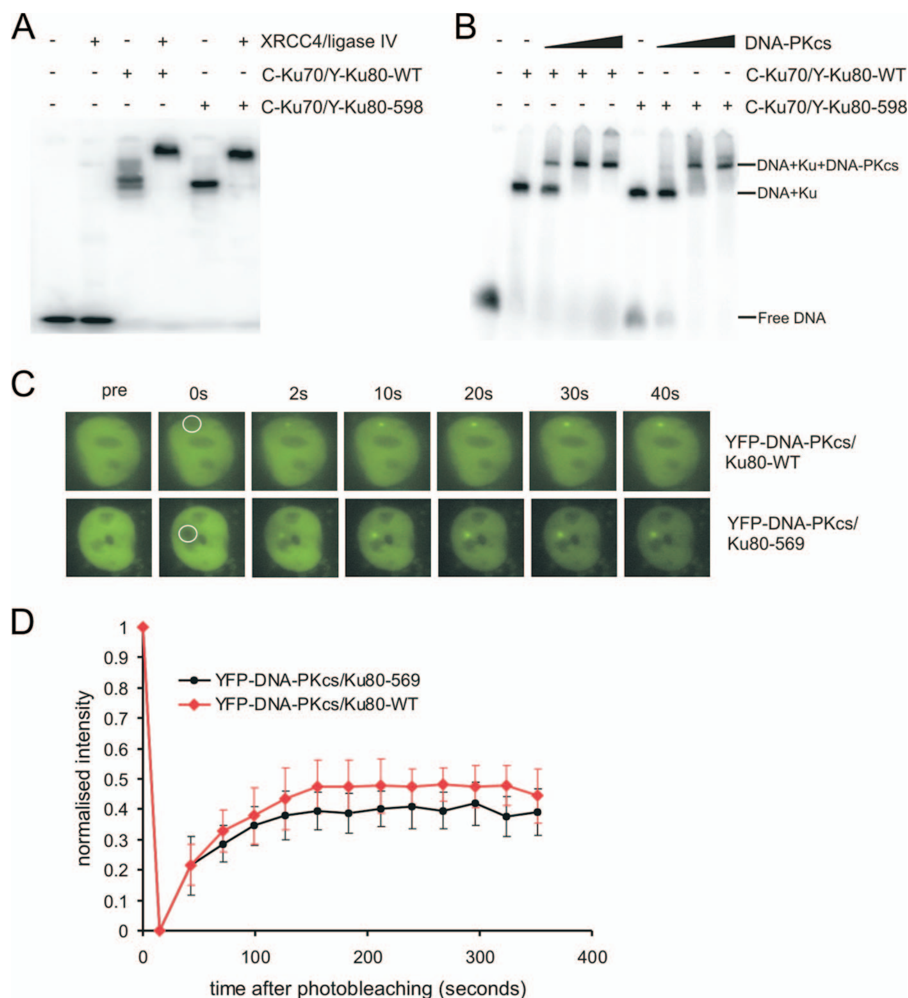

FIG. 2. The Ku80 carboxy terminus is not required for XRCC4/ligase IV or DNA-PK$_{CS}$ recruitment to DSBs. (A) EMSA analysis, demonstrating the ability of Ku70/Ku80-WT and Ku70/Ku80-598 to form DNA-bound complexes with XRCC4/ligase IV. (B) EMSA analysis, demonstrating the ability of Ku70/Ku80-WT and Ku70/Ku80-598 to form DNA-bound complexes with increasing concentrations of DNA-PK$_{CS}$ (30, 100, and 300 ng per reaction). (C) In vivo accumulation of YFP-DNA-PK$_{CS}$ at laser-induced DSB sites in Ku80-WT (V3) or Ku80-569 (complemented Xrs6) cells. The white circle at $t = 0$ indicates the irradiated spot. (D) FRAP analysis of YFP-DNA-PK$_{CS}$ at DSB sites in V3 (Ku80 competent CHO cells)- or Ku80-569-complemented Xrs6 (Ku80-deficient CHO cells).

Ku80. Immediately after DSB induction, accumulation of both wild-type and mutant Ku80 was observed in the damaged region (Fig. 1E). Introduction of DSBs with a pulsed near-infrared laser also resulted in the normal accumulation of mutant Ku80 protein (18; data not shown). From these findings we conclude that deletion of the carboxy terminus does not interfere with the in vivo recruitment of Ku80 to DSBs.

The Ku80 carboxy terminus is not required for recruitment of XRCC4/ligase IV or DNA-PK$_{CS}$ to DSBs. In a recent study, we presented evidence that the Ku70/80 heterodimer is indispensable for attraction of the XRCC4/ligase IV complex to DNA ends (18). We investigated whether the Ku80 carboxy terminus mediates this interaction by incubating a radiolabeled DNA fragment with purified XRCC4/ligase IV and either wild-type or mutant Ku70/80. EMSA analysis showed that a DNA-protein complex containing both XRCC4/ligase IV and Ku70/80 was formed equally efficiently when wild-type or mutant Ku70/80 was present in the reaction mixture (Fig. 2A). These results demonstrate that the Ku80 carboxy terminus does not mediate the interaction between DNA, Ku70/80, and XRCC4/ligase IV.

Because it has been previously suggested that the Ku80 carboxy terminus mediates the interaction between Ku70/80 and DNA-PK$_{CS}$ (12, 25), we performed an EMSA study with purified DNA-PK$_{CS}$ and either wild-type or mutant Ku70/80. As shown in Fig. 2B, the interaction between DNA, Ku70/80, and DNA-PK$_{CS}$ was only slightly diminished by deletion of the Ku80 carboxy terminus.

Subsequently, we studied the recruitment of DNA-PK$_{CS}$ to DSB sites in vivo. We therefore constructed cells that stably expressed YFP-DNA-PK$_{CS}$ and either wild-type or mutant Ku80. DSBs were introduced in the nucleus of these cells by laser microirradiation. Time-lapse imaging revealed that DNA-PK$_{CS}$ accumulated at DSB sites in both the Ku80 wild-type and the Ku80 mutant cells (Fig. 2C), whereas no DNA-PK$_{CS}$ accumulation was found in Ku80-deficient cells (28).

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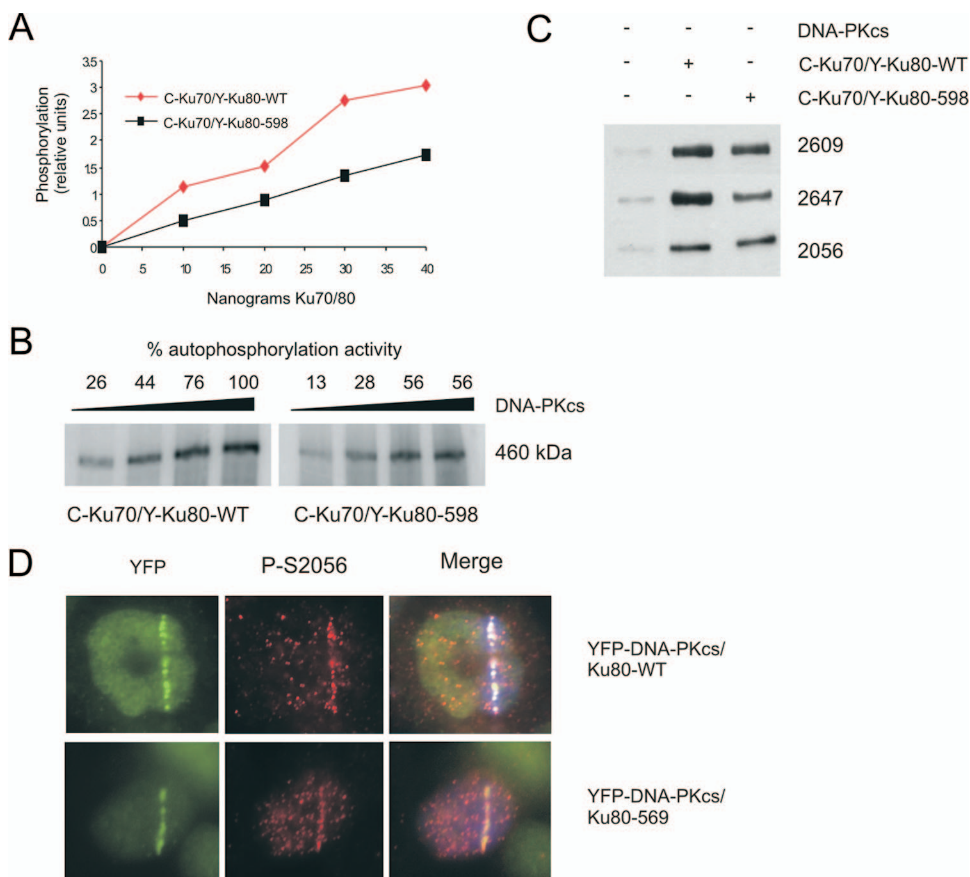

FIG. 3. The Ku80 carboxy terminus is not essential for DNA-PK$_{CS}$ kinase activity. (A) In vitro DNA-PK$_{CS}$ kinase activity in the presence of Ku70/Ku80-WT or Ku70/Ku80-598. Kinase activity is determined by measuring phosphorus incorporation into a p53-based peptide (A) or into the DNA-PK$_{CS}$ band (B). (C) In vitro autophosphorylation of DNA-PK$_{CS}$ residues 2056, 260, and 2647 in the presence of Ku70/Ku80-WT or Ku70/Ku80-598. (D) In vivo phosphorylation of the DNA-PK$_{CS}$ Ser2056 residue at DSB sites in V3 (Ku80-competent CHO cells) or Ku80-569-complemented Xrs6 (Ku80-deficient CHO cells). The phospho-specific signal colocalizes with YFP-DNA-PK$_{CS}$.

Evidently, deletion of the Ku80 carboxy terminus does not interfere with recruitment of DNA-PK$_{CS}$ to DSBs in vivo.

In order to examine whether the Ku80 carboxy terminus influences the stability of DNA-PK$_{CS}$ binding to DSBs, we photobleached the DSB region after maximal accumulation of YFP-DNA-PK$_{CS}$ and monitored the recovery of the fluorescence signal in time (FRAP). FRAP kinetics were similar in Ku80 wild-type and mutant cells, indicating that the Ku80 carboxy terminus does not contribute detectably to the stability of the DNA–DNA-PK$_{CS}$ complex in vivo (Fig. 2D).

The Ku80 carboxy terminus is not essential for DNA-PK$_{CS}$ kinase activity. Since DNA-PK$_{CS}$ recruitment to DSBs was not affected by deletion of the Ku80 carboxy terminus, we investigated whether the DNA-PK$_{CS}$ kinase activity might be compromised in a Ku mutant background. We first measured the DNA-PK$_{CS}$-mediated phosphorylation of a p53-based peptide in vitro. Mutant Ku80 activated the DNA-PK$_{CS}$ kinase to a level that was ca. 50% of the level observed with wild-type Ku80 (Fig. 3A). This demonstrates that, although reduced, in vitro activation of the DNA-PK$_{CS}$ kinase can still take place in the absence of the Ku80 carboxy terminus.

We then examined the ability of DNA-PK$_{CS}$ to autophosphorylate in the presence of mutant Ku80 protein. Therefore, either wild-type or mutant Ku70/80 was incubated with purified DNA-PK$_{CS}$, a linear DNA fragment and [γ-^{32}P]ATP. The reaction mixture was subsequently separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and phosphorylation of DNA-PK$_{CS}$ was detected by autoradiography. A radioactively labeled DNA-PK$_{CS}$ band appeared in both reaction mixtures, demonstrating that both wild-type and mutant Ku80 were able to support DNA-PK$_{CS}$ autophosphorylation (Fig. 3B). Overall, DNA-PK$_{CS}$ autophosphorylation in the mutant Ku80 reaction took place at a rate that is ca. 50% of the rate in the wild-type Ku80 reaction, very similar to the level of peptide phosphorylation observed in the previous assay.

In addition, we investigated whether mutation of the Ku80 protein interferes with the phosphorylation of specific DNA-PK$_{CS}$ autophosphorylation sites. Therefore, either wild-type or mutant Ku70/80 was incubated with purified DNA-PK$_{CS}$, and the reaction mixtures were separated by SDS-PAGE. Phosphorylation of the DNA-PK$_{CS}$ Ser2056, Thr2609, and Thr2647 residues (35) was subsequently determined by using phospho-specific antibodies (Fig. 3C). We found that all three residues were phosphorylated in the presence of both wild-type and mutant Ku80, but phosphorylation at Thr2647 was markedly

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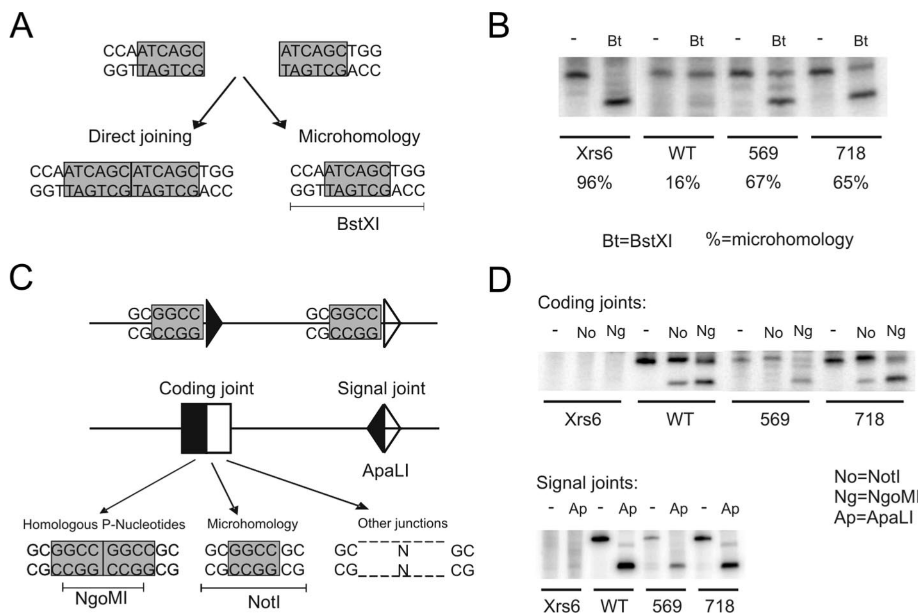

FIG. 4. Deletion of the Ku80 carboxy terminus alters end joining and V(D)J recombination characteristics. (A) Schematic depiction of the end-joining assay. Only the use of microhomology-mediated joining results in the formation of a BstXI site. The relative contribution of microhomology is estimated by analysis of the BstXI sensitivity of the joints. (B) End-joining assay in Xrs6 cells and (YFP-labeled) Ku80-WT-, Ku80-569-, or Ku80-718-complemented Xrs6 cells. Junctions were PCR amplified, and PCR products were digested with BstXI. (C) Schematic depiction of the V(D)J recombination assay. The formation of coding joints without loss of sequence or on a 4-bp microhomology results in the formation of an NgoMI or a NotI site, respectively. Only precise signal joints contain an ApaLI site. (D) V(D)J recombination assay, analyzing the formation and sequence of both coding joints and signal joints in Xrs6 cells and (YFP-labeled) Ku80-WT-, Ku80-569-, or Ku80-718-complemented Xrs6 cells.

reduced in reactions with Ku80-598 and Thr2609 was somewhat less efficiently phosphorylated by this Ku80 mutant. Interestingly, these sites are located within the ABCDE cluster that was previously reported to require phosphorylation for subsequent processing and joining reactions.

Next, we set out to investigate whether deletion of the Ku80 carboxy terminus impairs DNA-PK$_{CS}$ activation and autophosphorylation in vivo. We induced DSBs by laser microirradiation in cells that stably expressed YFP-DNA-PK$_{CS}$ and either wild-type or mutant Ku80. After maximal accumulation of DNA-PK$_{CS}$ at the DSB region, an immunofluorescence staining was performed with a phospho-specific antibody against the Ser2056 residue of the DNA-PK$_{CS}$ protein. We detected Ser2056 phosphorylation in both wild-type and mutant Ku80 cells, colocalizing with the YFP signal (Fig. 3D). Because the Ser2056 site is known to be autophosphorylated in response to DSBs (4, 6, 9, 10, 28), we conclude that DSB-induced activation and autophosphorylation of DNA-PK$_{CS}$ does not require the Ku80 carboxy terminus in vivo. Since phosphorylation at Thr2609 and Thr2647 is not exclusively done by DNA-PK, we did not investigate this activity in vivo (5, 35).

The Ku80 carboxy terminus stimulates Artemis-mediated DNA end processing. In order to explain why the Ku80 carboxy terminus has such a marked influence on radiation sensitivity, even though it is not an absolute requirement for activation of DNA-PK$_{CS}$ and interaction with XRCC4/ligase IV, we investigated the repair defect in Ku80 mutant cells in more detail. To that end, we performed plasmid recircularization assays, which enabled us to determine the relative use of direct and microhomology-mediated joining in ligation products (Fig. 4A, (32). Xrs6 cells, complemented with either wild-type or mutant Ku80, were transfected with the linearized pDVG94 plasmid, and joining products were subsequently analyzed. The relative level of microhomology use was determined by digestion with the restriction enzyme BstXI, which cleaved products that had been joined on a terminal 6-bp direct repeat sequence. Ku80-deficient Xrs6 cells were characterized by a shift to microhomology-mediated joining, which could be reversed to the normal low microhomology use by complementation with wild-type Ku80 (Fig. 4B). Complementation with the Ku80 deletion mutants resulted in an intermediate level of microhomology use, suggesting that the Ku80 mutants can partially rescue the repair defect of Xrs6 cells (Fig. 4B). These results demonstrate that the Ku80 carboxy terminus has a role in joining of compatible DNA ends, although deletion of this domain allows residual activity.

In addition, we performed an assay to measure the efficiency and accuracy of V(D)J recombination (Fig. 4C) (32). Cells were transfected with a V(D)J recombination substrate harboring two recombination signal sequences in direct orientation, allowing analysis of signal and coding joints on the same plasmid. Wild-type Ku80 efficiently restored both signal and coding joint formation in Xrs6 cells, whereas noncomplemented Xrs6 cells hardly formed any junctions (Fig. 4D). Cells complemented with mutant Ku80-569 and Ku80-598 formed markedly lower levels of coding and signal joints than wild-type Ku80 complemented cells but higher than noncomplemented Xrs6 cells (Fig. 4D and data not shown). Interestingly, complementation was more efficiently accomplished by Ku80-718 than by Ku80-569, suggesting that inhibition of coding and signal

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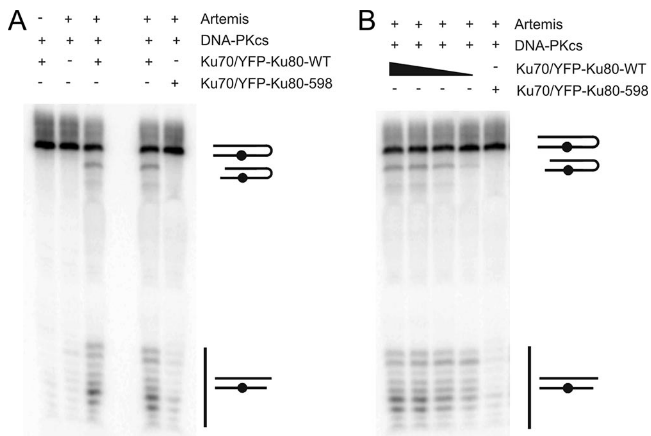

FIG. 5. The Ku80 carboxy terminus stimulates Artemis-mediated DNA end processing. (A) In vitro hairpin processing assay. In the left panel, a hairpin opening requires the presence of both Artemis and Ku70/80. In the right panel, the hairpin opening activity is significantly reduced when Ku70/Ku80-598 is used instead of Ku70/Ku80-WT. Equal amounts of Ku70/Ku80-598 and Ku70/Ku80-WT were used. (B) Titration of Ku70/Ku80-WT in the hairpin opening assay. Even when Ku70/Ku80-WT is used at a 10-fold-lower concentration than Ku70/Ku80-598, the hairpin opening is still markedly more prevalent in the Ku70/Ku80-WT reaction. From left to right, the Ku70/Ku80-WT concentrations were 10, 5, 2.5, and 1 ng/μl, while Ku70/Ku80-598 was present at 10 ng/μl.

joint formation is not solely caused by deletion of the carboxy-terminal 14 amino acids and that other elements in the carboxy terminus also contribute to efficient V(D)J recombination.

Signal joints were precise in both Ku80 wild-type and Ku80 mutant complemented cells, as verified by digestion with ApaLI. Interestingly, however, the sequence of the coding joints was quite different between the Ku80 wild-type and the Ku80 mutant complemented cells. The V(D)J recombination construct pDVG93 was designed to discriminate various types of joining by simple restriction enzyme digestion of the junctions (Fig. 4C). We previously found that Artemis-deficient patient fibroblasts showed a complete shift toward coding joints that were NgoMI sensitive (29). Cells complemented with Ku80 truncation mutants showed a similar shift toward NgoMI-sensitive junctions, the severity of which depended on the length of the deletion, suggesting that the Ku80 carboxy terminus contains elements that stimulate hairpin processing by the Artemis nuclease.

In order to obtain more evidence for a role for the Ku80 carboxy terminus in Artemis stimulation, we performed a hairpin processing assay. A radioactively labeled DNA hairpin substrate was incubated with purified Artemis, DNA-PK$_{CS}$ and either wild-type or mutant Ku70/80. DNA digestion products were subsequently analyzed by denaturing gel electrophoresis. In the wild-type Ku80 reaction, we observed both hairpin opening products and a cleavage product that was shortened by only a few base pairs (Fig. 5A). This latter activity most likely reflected Artemis-dependent trimming of the nonhairpin DNA end (39). Processing of the hairpin substrate was dependent on the presence of both Artemis and Ku70/80 (Fig. 5A, left panel). Replacing wild-type Ku70/80 with an equal amount of mutant Ku70/80 protein resulted in a drastic reduction in Artemis-mediated hairpin processing (Fig. 5A, right panel). Next, we titrated the concentration of wild-type Ku70/80 in the reaction mixture down to a level that was 10-fold lower than that used in the mutant Ku70/80 reaction. We still observed a markedly higher hairpin processing activity in the wild-type Ku80 reaction (Fig. 5B). Collectively, these data demonstrate that deletion of the Ku80 carboxy terminus affects autophosphorylation at specific sites in DNA-PK$_{CS}$, resulting in a drastic reduction of end joining and Artemis-mediated DNA end processing, which probably explains the radiosensitivity of cells harboring such a mutation.

DISCUSSION

Our experiments demonstrated that the Ku80 carboxy terminus is dispensable for binding of the Ku70/80 heterodimer to DNA termini and for subsequent recruitment and activation of DNA-PK$_{CS}$. The observation that deletion of the Ku80 carboxy terminus does not interfere with DNA binding of the Ku70/80 molecule corroborates well with previous studies which suggested that Ku70/80 DNA binding is mediated by the amino-terminal and central regions of both Ku70 and Ku80 (33). However, our finding that deletion of the Ku80 carboxy terminus does not abolish DNA-PK$_{CS}$ recruitment and activation in vivo is dissimilar from the conclusions of earlier work (11, 12, 25).

Falck et al. reported that the carboxy-terminal 14 amino acids of Ku80 are responsible for recruitment and activation of the DNA-PK$_{CS}$ kinase (11). This region is characterized by a "PIKK interaction" motif that is also conserved in NBS1 and ATRIP, proteins that are responsible for the DSB-induced activation of two DNA-PK$_{CS}$-related kinases (ATM and ATR, respectively). Our findings show that this motif is not the sole factor that mediates DNA-PK$_{CS}$ activation. We observed that deletion of the Ku80 carboxy terminus results in an ca. 50% decreased DNA-PK$_{CS}$ kinase activity in vitro, suggesting that the carboxy terminus of Ku80 contributes to, but is not essential for the activation of DNA-PK$_{CS}$ (Fig. 3A and B). Interestingly, DNA-PK$_{CS}$ autophosphorylation was differentially affected at Ser2056 (which was phosphorylated to wild-type levels) and Thr2609 and Thr2647 (where phosphorylation was reduced). This may explain why Falck et al. concluded that DNA-PK activity was severely affected, since they only investigated Thr2609 phosphorylation. Our observations suggest that the balance between phosphorylation at the ABCDE and PQR clusters determines the relative accessibility of DNA ends for processing and joining enzymes. However, the occurrence of DNA-PK$_{CS}$ kinase-mediated autophosphorylation in Ku80 mutant cells clearly indicates that the presence of the conserved 14-amino-acid region or even the entire carboxy terminus is not an absolute necessity for DNA-PK$_{CS}$ activation.

Since in vivo DNA-PK$_{CS}$ recruitment, activation, and autophosphorylation requires the presence of Ku80 (28) but not the Ku80 carboxy terminus, we infer that these processes must be governed by domains in the amino-terminal or central regions of the Ku80 molecule, possibly in cooperation with the 14-amino-acid PIKK interaction motif (Fig. 6). This notion is supported by the fact that complementation of Xrs6 cells with Ku80 deletion mutants partially rescues the end joining and

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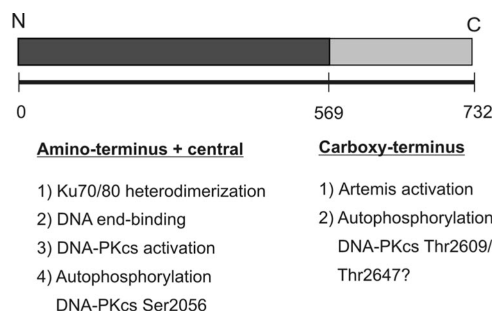

FIG. 6. Different roles for the Ku80 domains. This model proposes that the amino terminus and/or the central region of Ku80 are necessary to effectively recruit and activate DNA-PK$_{CS}$. The Ku80 carboxy terminus stimulates autophosphorylation of the Thr2609 and possibly the Thr 2647 residues of DNA-PK$_{CS}$, as well as activation of the Artemis endonuclease.

V(D)J deficiency of this cell line (Fig. 4B and D). Cryo-electron microscopy studies on Ku/DNA-PK$_{CS}$ complexes revealed a rather extensive interface between Ku and DNA-PK$_{CS}$, providing additional evidence that the Ku80 carboxy terminus only mediates part of the interaction between Ku and DNA-PK$_{CS}$ (26).

Two recent studies showed that the conserved motif in NBS1 is not solely responsible for ATM activation and that different domains within the NBS1 molecule govern different ATM-mediated responses (8, 27). We believe that the activation of DNA-PK$_{CS}$ may be mediated by Ku80 in a similar fashion as the activation of ATM by NBS1, i.e., as a cooperative effort of different domains within the activator molecule.

The absence of an active Artemis endonuclease is likely to effectively stall or inhibit the correct processing of a subset of incompatible DNA ends, including the hairpin coding ends that are formed during V(D)J recombination. The fraction of nonrepairable DSBs in the absence of Artemis varies between 10 and 20% for most qualities of ionizing radiation, suggesting that this represents the fraction of IR-induced DNA ends that require Artemis for proper processing, although the nature of these DSBs is not yet clear (23, 34).

Hairpin opening and endonuclease activity may require both Artemis and DNA-PK (17). Therefore, the Ku80 carboxy terminus could exert its stimulating influence on either component of the DNA-PK$_{CS}$/Artemis complex, and it is tempting to speculate that the Ku/DNA-PK$_{CS}$/Artemis triad requires the presence of the Ku80 carboxy terminus to assume an active configuration. Our data suggest that the Ku80 carboxy terminus mediates subtle features of DNA-PK$_{CS}$ (auto)phosphorylation, which in turn may influence DNA processing by Artemis (14). The exact nature of these interactions, however, will need further experimentation. The availability of Ku80 mutants that are severely affected in their ability to mediate such interactions will help to unravel the details of this intricate interplay between various proteins at DNA ends.

In conclusion, our experiments suggest that the importance of the Ku80 carboxy terminus for NHEJ-mediated DSB repair may be partially dependent upon interaction with or activation of DNA-PK$_{CS}$, but to a larger extent upon correct autophosphorylation and activation of the DNA-PK$_{CS}$/Artemis complex. This model comprehensively explains both the IR-sensitivity and the impaired V(D)J recombination activity that result from deletion of the Ku80 carboxy terminus.

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REFERENCES

1. **Chan, D. W., B. P. Chen, S. Prithivirajsingh, A. Kurimasa, M. D. Story, J. Qin, and D. J. Chen.** 2002. Autophosphorylation of the DNA-dependent protein kinase catalytic subunit is required for rejoining of DNA double-strand breaks. Genes Dev. **16:**2333–2338.
2. **Chan, D. W., C. H. Mody, N. S. Ting, and S. P. Lees-Miller.** 1996. Purification and characterization of the double-stranded DNA-activated protein kinase, DNA-PK, from human placenta. Biochem. Cell Biol. **74:**67–73.
3. **Chappell, C., L. A. Hanakahi, F. Karimi-Busheri, M. Weinfeld, and S. C. West.** 2002. Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining. EMBO J. **21:**2827–2832.
4. **Chen, B. P., D. W. Chan, J. Kobayashi, S. Burma, A. Asaithamby, K. Morotomi-Yano, E. Botvinick, J. Qin, and D. J. Chen.** 2005. Cell cycle dependence of DNA-dependent protein kinase phosphorylation in response to DNA double strand breaks. J. Biol. Chem. **280:**14709–14715.
5. **Chen, B. P., N. Uematsu, J. Kobayashi, Y. Lerenthal, A. Krempler, H. Yajima, M. Lobrich, Y. Shiloh, and D. J. Chen.** 2007. Ataxia telangiectasia mutated (ATM) is essential for DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. J. Biol. Chem. **282:**6582–6587.
6. **Chen, B. P., N. Uematsu, J. Kobayashi, Y. Lerenthal, A. Krempler, H. Yajima, M. Lobrich, Y. Shiloh, and D. J. Chen.** 2006. ATM is essential for DNA-pkcs phosphorylations at T2609 cluster upon DNA double strand break. J. Biol. Chem. **282:**6582–6587.
7. **Denekamp, J., G. F. Whitmore, and P. Jeggo.** 1989. Biphasic survival curves for XRS radiosensitive cells: subpopulations or transient expression of repair competence? Int. J. Radiat. Biol. **55:**605–617.
8. **Difilippantonio, S., A. Celeste, M. J. Kruhlak, Y. Lee, M. J. Difilippantonio, L. Feigenbaum, S. P. Jackson, P. J. McKinnon, and A. Nussenzweig.** 2007. Distinct domains in Nbs1 regulate irradiation-induced checkpoints and apoptosis. J. Exp. Med. **204:**1003–1011.
9. **Ding, Q., Y. V. Reddy, W. Wang, T. Woods, P. Douglas, D. A. Ramsden, S. P. Lees-Miller, and K. Meek.** 2003. Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair. Mol. Cell. Biol. **23:**5836–5848.
10. **Douglas, P., G. P. Sapkota, N. Morrice, Y. Yu, A. A. Goodarzi, D. Merkle, K. Meek, D. R. Alessi, and S. P. Lees-Miller.** 2002. Identification of in vitro and in vivo phosphorylation sites in the catalytic subunit of the DNA-dependent protein kinase. Biochem. J. **368:**243–251.
11. **Falck, J., J. Coates, and S. P. Jackson.** 2005. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature **434:**605–611.
12. **Gell, D., and S. P. Jackson.** 1999. Mapping of protein-protein interactions within the DNA-dependent protein kinase complex. Nucleic Acids Res. **27:**3494–3502.
13. **Gellert, M.** 2002. V(D)J. recombination: RAG proteins, repair factors, and regulation. Annu. Rev. Biochem. **71:**101–132.
14. **Goodarzi, A. A., Y. Yu, E. Riballo, P. Douglas, S. A. Walker, R. Ye, C. Harer, C. Marchetti, N. Morrice, P. A. Jeggo, and S. P. Lees-Miller.** 2006. DNA-PK autophosphorylation facilitates Artemis endonuclease activity. EMBO J. **25:**3880–3889.
15. **Helleday, T., J. Lo, D. C. van Gent, and B. P. Engelward.** 2007. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair **6:**923–935.
16. **Lieber, M. R., H. Lu, J. Gu, and K. Schwarz.** 2008. Flexibility in the order of action and in the enzymology of the nuclease, polymerases, and ligase of vertebrate non-homologous DNA end joining: relevance to cancer, aging, and the immune system. Cell Res. **18:**125–133.
17. **Ma, Y., U. Pannicke, K. Schwarz, and M. R. Lieber.** 2002. Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J. recombination. Cell **108:**781–794.
18. **Mari, P. O., B. I. Florea, S. P. Persengiev, N. S. Verkaik, H. T. Bruggenwirth, M. Modesti, G. Giglia-Mari, K. Bezstarosti, J. A. Demmers, T. M. Luider,**

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A. B. Houtsmuller, and D. C. van Gent. 2006. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. Proc. Natl. Acad. Sci. USA **103:**18597–18602.
19. **Meek, K., S. Gupta, D. A. Ramsden, and S. P. Lees-Miller.** 2004. The DNA-dependent protein kinase: the director at the end. Immunol. Rev. **200:**132–141.
20. **Modesti, M., M. S. Junop, R. Ghirlando, M. van de Rakt, M. Gellert, W. Yang, and R. Kanaar.** 2003. Tetramerization and DNA ligase IV interaction of the DNA double-strand break repair protein XRCC4 are mutually exclusive. J. Mol. Biol. **334:**215–228.
21. **Moshous, D., I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, A. Fischer, and J. P. de Villartay.** 2001. Artemis, a novel DNA double-strand break repair/V(D)J. recombination protein, is mutated in human severe combined immune deficiency. Cell **105:**177–186.
22. **Ono, M., P. W. Tucker, and J. D. Capra.** 1994. Production and characterization of recombinant human Ku antigen. Nucleic Acids Res. **22:**3918–3924.
23. **Riballo, E., M. Kuhne, N. Rief, A. Doherty, G. C. Smith, M. J. Recio, C. Reis, K. Dahm, A. Fricke, A. Krempler, A. R. Parker, S. P. Jackson, A. Gennery, P. A. Jeggo, and M. Lobrich.** 2004. A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. Mol. Cell **16:**715–724.
24. **Rooney, S., J. Sekiguchi, C. Zhu, H. L. Cheng, J. Manis, S. Whitlow, J. DeVido, D. Foy, J. Chaudhuri, D. Lombard, and F. W. Alt.** 2002. Leaky Scid phenotype associated with defective V(D)J. coding end processing in Artemis-deficient mice. Mol. Cell **10:**1379–1390.
25. **Singleton, B. K., M. I. Torres-Arzayus, S. T. Rottinghaus, G. E. Taccioli, and P. A. Jeggo.** 1999. The C terminus of Ku80 activates the DNA-dependent protein kinase catalytic subunit. Mol. Cell. Biol. **19:**3267–3277.
26. **Spagnolo, L., A. Rivera-Calzada, L. H. Pearl, and O. Llorca.** 2006. Three-dimensional structure of the human DNA-PKcs/Ku70/Ku80 complex assembled on DNA and its implications for DNA DSB repair. Mol. Cell **22:**511–519.
27. **Stracker, T. H., M. Morales, S. S. Couto, H. Hussein, and J. H. Petrini.** 2007. The carboxy terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. Nature **447:**218–221.
28. **Uematsu, N., E. Weterings, K. Yano, K. Morotomi-Yano, B. Jakob, G. Taucher-Scholz, P. O. Mari, D. C. van Gent, B. P. Chen, and D. J. Chen.** 2007. Autophosphorylation of DNA-PK_{CS} regulates its dynamics at DNA double-strand breaks. J. Cell Biol. **177:**219–229.
29. **van der Burg, M., N. S. Verkaik, A. T. den Dekker, B. H. Barendregt, I. Pico-Knijnenburg, I. Tezcan, J. J. vanDongen, and D. C. van Gent.** 2007. Defective Artemis nuclease is characterized by coding joints with microhomology in long palindromic-nucleotide stretches. Eur. J. Immunol. **37:**3522–3528.
30. **van Gent, D. C., J. H. Hoeijmakers, and R. Kanaar.** 2001. Chromosomal stability and the DNA double-stranded break connection. Nat. Rev. Genet. **2:**196–206.
31. **van Gent, D. C., and M. van der Burg.** 2007. Non-homologous end-joining, a sticky affair. Oncogene **26:**7731–7740.
32. **Verkaik, N. S., R. E. Esveldt-van Lange, D. van Heemst, H. T. Bruggenwirth, J. H. Hoeijmakers, M. Z. Zdzienicka, and D. C. van Gent.** 2002. Different types of V(D)J. recombination and end-joining defects in DNA double-strand break repair mutant mammalian cells. Eur. J. Immunol. **32:**701–709.
33. **Walker, J. R., R. A. Corpina, and J. Goldberg.** 2001. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature **412:**607–614.
34. **Wang, J., J. M. Pluth, P. K. Cooper, M. J. Cowan, D. J. Chen, and S. M. Yannone.** 2005. Artemis deficiency confers a DNA double-strand break repair defect and Artemis phosphorylation status is altered by DNA damage and cell cycle progression. DNA Repair **4:**556–570.
35. **Weterings, E., and D. J. Chen.** 2007. DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys? J. Cell Biol. **179:**183–186.
36. **Weterings, E., and D. J. Chen.** 2008. The endless tale of non-homologous end-joining. Cell Res. **18:**114–124.
37. **Weterings, E., N. S. Verkaik, H. T. Bruggenwirth, J. H. Hoeijmakers, and D. C. van Gent.** 2003. The role of DNA dependent protein kinase in synapsis of DNA ends. Nucleic Acids Res. **31:**7238–7246.
38. **Whitmore, G. F., A. J. Varghese, and S. Gulyas.** 1989. Cell cycle responses of two X-ray sensitive mutants defective in DNA repair. Int. J. Radiat Biol. **56:**657–665.
39. **Yannone, S. M., I. S. Khan, R. Z. Zhou, T. Zhou, K. Valerie, and L. F. Povirk.** 2008. Coordinate 5′ and 3′ endonucleolytic trimming of terminally blocked blunt DNA double-strand break ends by Artemis nuclease and DNA-dependent protein kinase. Nucleic Acids Res. **36:**3354–3365.

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