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Distinct roles of XRCC4 and Ku80 in non-homologous end-joining of endonuclease- and ionizing radiation-induced DNA double-strand breaks

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ABSTRACT

Non-homologous end-joining (NHEJ) of DNA double-strand breaks (DSBs) is mediated by two protein complexes comprising Ku80/Ku70/DNA-PKcs/Artemis and XRCC4/LigaseIV/XLF. Loss of Ku or XRCC4/LigaseIV function compromises the rejoining of radiation-induced DSBs and leads to defective V(D)J recombination. In this study, we sought to define how XRCC4 and Ku80 affect NHEJ of site-directed chromosomal DSBs in murine fibroblasts. We employed a recently developed reporter system based on the rejoining of I-SceI endonuclease-induced DSBs. We found that the frequency of NHEJ was reduced by more than 20-fold in XRCC4−/− compared to XRCC4+/+ cells, while a Ku80 knock-out reduced the rejoining efficiency by only 1.4-fold. In contrast, lack of either XRCC4 or Ku80 increased end degradation and shifted repair towards a mode that used longer terminal microhomologies for rejoining. However, both proteins proved to be essential for the repair of radiation-induced DSBs. Thus, I-SceI-induced breaks may resemble DSBs arising during normal DNA metabolism and mouse development. The removal of these breaks likely has different genetic requirements than the repair of radiation-induced DSBs.

INTRODUCTION

DNA double-strand breaks (DSBs) represent the most serious DNA lesion, which, if not adequately repaired, can lead to cell death through the generation of lethal chromosomal aberrations. Alternatively, inadequately repaired DSBs may give rise to potentially carcinogenic mutations or chromosomal rearrangements. In mammalian cells, non-homologous end-joining (NHEJ) is the principal pathway for the removal of DSBs throughout the entire cell cycle. NHEJ relies on a limited number of core proteins that are sufficient to execute DSB repair in vitro (1,2). The heterodimer of Ku70 and Ku80 recognizes and binds DNA ends and recruits the catalytic subunit of the DNA-dependent kinase (DNA-PKcs), together forming the DNA-PK holoenzyme. Ku proteins and DNA-PKcs are both capable of tethering DNA ends (3–5), with Ku translocating internally upon binding of DNA-PKcs to the DNA end (6). Prior to ligation, the DNA ends need to be trimmed for proper annealing. At least a fraction of DSB ends is tailored by the Artemis...
endonucleases are not yet defined. The polymerases Pol\(\mu\) and Pol\(\lambda\) likely replenish small sequence gaps (8,9). The fill-in synthesis appears to be tightly coupled to the ligation of DNA ends (2,8). The latter step is performed by DNA ligase IV (LigIV) together with its obligatory cofactor XRCC4 (10,11). Another partner involved in the ligation step \textit{in vivo} has been recently identified as the XRCC4-like factor (\textit{=} XLF, Cernunnos) (12,13). The ligation complex is recruited by and interacts with Ku and DNA-PKcs (14–16).

Cells deficient in any of the NHEJ core proteins display pronounced hypersensitivity to ionizing radiation (IR) and a reduced ability to rejoin IR-induced DSBs (17). The NHEJ core proteins are also required for V(D)J recombination and class-switch recombination (18–20). Deficiency of either of these proteins leads to severe clinical immunodeficiency in mice and humans (21–25). Further, NHEJ deficiencies in mice are associated with impaired neurogenesis and growth delay (25–27). Defective NHEJ also causes gross chromosomal aberrations, genomic instability and lymphomagenesis (28,23). In humans, however, defective NHEJ has not yet been extensively linked to malignancy (29–32).

Genetic knock-out of XRCC4 but not of Ku80 in mice leads to embryonic death, suggesting that XRCC4 function is critical for the removal of DSBs that arise during development (22,23). Interestingly, this differential importance of XRCC4 and Ku80 for the DSB repair efficiency is generally not well reflected in biochemical and extrachromosomal end-joining assays (33–36). Furthermore, loss of XRCC4 or Ku80 causes IR hypersensitivity that is of similar severity.

Chromosomal plasmid assays that employ the rare-cutting I-SceI endonuclease have been employed successfully to elucidate the genetic determinants and molecular mechanisms of homologous recombination (37,38). Recently, others and we have applied these assays to NHEJ as well (39–41). In a report by Lopez and colleagues (39), mutation of Ku80 had surprisingly little if any effect on the rejoining of non-complementary ends generated by cleavage of two inverted I-SceI recognition sites spaced some kb apart. However, it cannot be excluded that residual Ku80 activity in the xrs6 CHO cells was sufficient for DSB rejoining in that assay. The importance of XRCC4/LigIV for the rejoining of site-directed chromosomal breaks was not studied and has remained unknown.

In the present study, we therefore investigated the roles of XRCC4 and Ku80, as the respective representatives of the XRCC4/XLF/LigIV and Ku/DNA–PKcs/Artemis complex, in the rejoining of I-SceI endonuclease-induced DSBs. We report the chromosomal repair phenotype of XRCC4 null mouse cells, which is characterized by a more than 20-fold reduction of NHEJ proficiency, increased end-degradation, and an increase in microhomology length used for joining of ends. Strikingly, knock-out of Ku80 resulted only in a mild I-SceI end-joining defect (1.4-fold), while having an impact on repair fidelity that was similar to the loss of XRCC4.

Figure 1. NHEJ reporter construct. (A) Illustration of the reporter assay. See text for details. (B) Double-stranded sequence flanking both I-SceI recognition sites (bold), which are placed in inverse orientation: Simultaneous cleavage of both sites with pop-out of the intervening sequence generates two non-compatible cohesive ends. A single I-SceI cleavage generates compatible cohesive ends that can be directly religated. The artificial start codon is underscored. Figure is adopted from (41).

**MATERIALS AND METHODS**

**Cells**

Mouse embryonic fibroblasts (MEFs) lacking either Ku80 or the XRCC4 (28, 23, kindly gifted by A. Nussenzweig and F. Alt) and the respective parental strains (all strains were p53\(−\)/\(−\)) were cultured in DMEM medium supplemented with 15% FCS, 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO\(_2\). The presence or absence of XRCC4 and Ku80 was verified by Western blotting (Supplementary Figure S1). To harvest clones grown in selection medium (either Puromycin or XHATM, see below) in T-175 plastic culture flasks (Greiner, Germany), a 75W soldering iron (ERSA Multisprint, ERSA, Germany) was used to melt a 1 cm hole directly above each individual colony through which careful micro-trypsinization (10\(\mu\)l Tryptsin-EDTA, GibCO-Invitrogen) was possible. Only those clones were chosen that grew in sufficient distance (> 1cm) to its proximate neighbor colony to avoid cross contamination. The individual clones were transferred to microwell plates and further expanded.

**NHEJ reporter substrate**

The generation of the pPHW2 plasmid was described previously (40). Induction of DSBs by the I-SceI endonuclease and rejoining by NHEJ lead to gpt translation and resistance to XHATM-containing selection medium (Figure 1). Here, 0.5\(\mu\)g of the pPHW2 was linearized with PvuI and electroporated into \(10^6\) cells. Cells were grown for 2–3 weeks in selection medium (0.5\(\mu\)g/ml puromycin, Sigma) to obtain clones with stable integration of pPHW2. This was verified by PCR using the primer pair ATGTTGCAGATCCATGCACG and
AATACGACGCCATATCCC, yielding a 400-bp fragment only in clones with an integrated repair substrate. Single copy integration was verified by sequencing of repair products (see below).

NHEJ assay

DSBs were induced by transfecting 3 x 10^6 cells carrying the pPHW2 plasmid with 50 µg of pCMV-3×NLS-I-SceI (kindly provided by M. Jasin) or a control plasmid (40). To allow for I-SceI expression and end-joining to proceed, cells were grown for 48 h in non-selective medium. Cells were then replated at appropriate densities between 10^5 and 10^6 per 175 cm² tissue culture flasks and grown for 2–3 weeks in selection medium containing XHATM (xanthine, hypoxanthine, aminopterin, thymidine and mycophenolic acid at 10, 13.6, 0.17, 3.87 and 10 µg/ml respectively; all Sigma). The relative NHEJ frequency was derived from the number of XHATM-resistant colonies per number of seeded cells. This frequency was corrected for the plating efficiency of control cells grown in non-selective medium and normalized to the transfection efficiency, ranging between 0.53 and 0.56 for Ku80 cells and 0.43 and 0.48 for XRCC4 cells.

Repair product analysis

Individual XHATM-resistant colonies were harvested, expanded and genomic DNA was subjected to sequencing across the repair junction using the primers agctattccagaagtagtgaggag (forward) and gtgatcgtagctggaaatacaaac (reverse), an automated sequencer (ABI 3100, Applied Biosystems-Hitachi) and Big-Dye technology.

Clonogenic cell survival and overall DSB rejoining after irradiation

Cells were irradiated with X-ray doses of up to 8 Gy and seeded for colony formation as described (42). For measuring of DSB repair, cells were irradiated with 50 Gy and rejoining of DSBs was measured at different time intervals after irradiation by neutral constant-field gel electrophoresis as described (42).

Statistics

Experiments were repeated at least three times, and data are presented as mean ± SEM. Statistical analysis, data fitting and graphics were performed by means of the Prism 4.0 computer program (GraphPad Software, San Diego, USA).

RESULTS

To elucidate the roles of XRCC4 and Ku80 in chromosomal NHEJ, we made use of a recently developed I-SceI reporter assay that has been described elsewhere (40,41). Briefly, the plasmid substrate, pPHW2, contains an artificial translational start sequence inserted between an early SV40 promoter and the bacterial gpt gene (Figure 1A). The associated artificial open reading frame is shifted by 1 bp against the downstream gpt ORF and is dominant over the gpt start site, hence, preventing gpt translation. Two I-SceI recognition sites flank the artificial ATG site. Simultaneous cleavage at both I-SceI sites results in frequent loss, i.e. pop-out, of the 34 bp-sequence containing the artificial ATG. NHEJ of the resulting DNA ends reconstitutes translation of the original gpt ORF, thereby allowing the detection of recombinants as colonies growing in XHATM selection medium. The tandem I-SceI sites are inverted, so that I-SceI cleavage results in two of non-complementary 3' single-stranded overhangs of 4 bases. These ends require modification prior to ligation (Figure 1B), which is a typical feature of repair via NHEJ (2, 34, 35, 41).

A hallmark of this plasmid design is that it does not select for specific NHEJ products, i.e. the pop-out mechanism allows for the detection of a broad variety of sequence alterations that range from single nucleotide alterations to deletions of up to 100 bp at the I-SceI sites. Since the 34-bp pop-out leaves the DNA ends in close proximity (within one nucleosome), the rejoining mechanism is likely representative of the repair of a single DSB. In addition, the assay can monitor a variety of repair events associated with sequence alterations after a single I-SceI cleavage (for details see below and Ref. (40,41)). In contrast, a direct ligation of an individually cleaved I-SceI site, which likely occurs in the majority of cells, cannot be detected as it does not alter the plasmid sequence. Finally, DSBs induced in the S/G2-phases of the cell cycle may also be repaired via error-free homologous recombination with the sister chromatid as a donor, which likewise does not alter the wild-type sequence and thus remains undetectable.

Lack of XRCC4 reduces the efficiency and fidelity of NHEJ of I-SceI-induced chromosomal DSBs

The pPHW2 reporter was stably integrated as a single copy into the genome of MEFs derived from XRCC4+/+ or XRCC4−/− mice (23). Subsequently, cells were transiently transfected with the I-SceI expression plasmid and incubated for 3 weeks in selective medium for colony formation. Four independent XRCC4+/+ clones carrying pPHW2 showed a mean NHEJ frequency of 0.034 (SEM, ±0.01) (Figure 2A, Supplementary Table S1). Knock-out of XRCC4 in another four clones drastically reduced the NHEJ efficiency on average to 0.0015 (±0.00045), corresponding to a 23-fold suppression compared to the parental cells (P < 0.0001). Of note, the data indicate that a small fraction of DSBs was still rejoined in the absence of a functional XRCC4/LigIV complex.

The persistence of non-ligated DNA ends in XRCC4−/− cells may lead to increased end degradation and deletion formation. To address this possibility, we raised individual XHATM-resistant clones after induction of I-SceI breaks and performed DNA sequencing across the repair junctions (see Supplementary Figure S2). XRCC4+/+ cells showed minimal end resections of 0–4 nt, which were restricted to the 3' overhangs (Figure 2B). Rejoining was mediated exclusively by 1–2 nt of A/T microhomologies present in the overhangs. In contrast, this high-fidelity repair phenotype was absent in
 XRCC4−/− cells. In these cells, all individual repair products revealed large deletions ranging from 10 to 76 bp (Figure 2B and C). The mean deletion length was 0.25 bp (±0.2) and 31 bp (±2.2) in XRCC4+/+ and XRCC4−/− cells, respectively (Mann–Whitney test, \( P < 0.0001 \)). In addition to reduced repair efficiency and fidelity, XRCC4−/− cells frequently changed to a repair mode that employed longer microhomologies for the annealing of ends, i.e. 3–5 nt, which never occurred in XRCC4+/+ cells (Fisher’s exact test, \( P = 0.049 \)) (Figure 2D). End-joining along 1–2 nt of microhomology was observed in only 35% of repair events in XRCC4−/− cells.

**Lack of Ku80 reduces the fidelity but not the efficiency of NHEJ**

To compare the repair phenotype of XRCC4−/− MEFs with the consequences of losing Ku80, we integrated pPHW2 into an isogenic pair of Ku80+/+ and Ku80−/− MEFs (28). We found that lack of Ku80 only led to a statistically non-significant reduction of NHEJ, i.e. by a factor of 1.4 (\( P = 0.6 \)) (Figure 3A, Supplementary Table S1), which markedly contrasts with the more than 20-fold reduced rejoining ability of XRCC4−/− cells. We considered the possibility that extensive deletions (>100 bp) arising from the cleaved I-SceI sites could have affected the adjacent promoter or gpt reporter gene in the XRCC4−/− cells, thereby leading to XHATM sensitivity and low rejoining frequencies. We, therefore, plated cells following I-SceI break induction into non-selective medium for colony formation. We observed that colony survival was significantly reduced only in XRCC4−/− but not in Ku80-deficient cells when compared to the transfection of a pNeo control plasmid (Supplementary Figure S3). These data indicate that XRCC4−/− cells suffer from a high frequency of lethal unrejoined breaks while Ku80−/− are widely capable of resealing I-SceI breaks.

The absence of an effect on rejoining efficiency in Ku80−/− cells does not preclude the possibility of an alteration in the fidelity of repair. We thus proceeded to
perform DNA sequencing on individual MEF clones following I-SceI break induction. In Ku80+/+ MEFs, we observed a spectrum of repair products similar although not identical to the repair phenotype of XRCC4+/+ cells (Figure 3B), which is likely attributable to the different mouse strains used. As in the XRCC4−/− cells, we detected an increase in deletion size in Ku80−/− MEFs compared to wild-type cells (Figure 3C). In Ku80+/+ cells, 42% of junctions showed small deletions of 4 bp or less, but only 10% of these high-fidelity events were detected in knock-out cells. Accordingly, deletions in Ku80-deficient cells were significantly longer than in the wild-type cells, i.e., a mean of 30 bp (±4) versus 18 bp (±3), respectively (Mann–Whitney test, P = 0.005). In addition, Ku80−/− cells used significantly more often microhomologies of 4–5 nt than the wild-type cells (Figure 3D, P = 0.005), suggesting that loss of Ku80, similar to XRCC4, changes the mechanism of rejoining. Interestingly, the percentage of junctions mediated by 1–2 nt microhomology, which appeared to be a feature of XRCC4-mediated NHEJ, was virtually identical in Ku80+/+ and Ku80−/− cells (~50% of all events).

We noticed that the repair spectrum of XRCC4+/+ and Ku80+/+ MEFs was not identical, which is likely due to the different origin of the respective mouse strains (Figures 2 and 3) (23,28). To exclude that the less precise baseline NHEJ activities seen in the Ku80+/+ cells have masked the impact of losing Ku80, we repeated the experiments in K1 wild-type and Ku80-mutant CHO cells. Loss of Ku80 function only led to a slightly reduced NHEJ capacity, i.e. from 0.042 to 0.031 (Supplementary Table S2), which confirmed the observed mild defect in Ku80−/− MEFs compared to Ku80 cells (see Figure 3A).

Taken together, our data reveal a reduced fidelity of NHEJ and preferred usage of longer microhomologies in both repair-deficient strains; however, only loss of XRCC4 significantly reduced the efficiency of rejoining.

**Lack of XRCC4 or Ku80 enhances error-prone rejoining of complementary DNA ends**

Previous studies with extrachromosomal substrates suggested that Ku80 and LigIV are involved in the precise ligation of complementary DNA ends (43–45).
As outlined above, complementary ends are created when only one I-SceI site is cleaved. Error-free religation does not alter the DNA sequence and cannot be detected directly. However, if precise end-ligation fails, sequence alterations that abolish the dominant function of the artificial start codon may occur (41). Examples of single-cleavage events are included in Figures 2B and 3B where the artificial ATG was either deleted (Sequences 4/4, 14/5, 14/13, 17/10) or shifted in frame with the original start codon (Sequences 52/11, 52/13), either of which restored gpt translation and resistance to XHATM.

We next analyzed the subpopulation of repair products arising from cleavage at individual I-SceI sites in more detail to determine whether loss of either XRCC4 or Ku80 had compromised the rate of precise endligation, thereby leading to sequence alteration upon repair of the ends (i.e. error-prone repair). Specifically, we estimated the fraction of error-prone repair events at individual I-SceI sites among all detectable repair events, i.e. individual I-SceI repair plus repair following pop-out events. In XRCC4+/+ cells, the portion of repair events at individual I-SceI sites was small (11%) (Supplementary Figure S2B), suggesting that the vast majority of these breaks was closed by precise religation. In contrast, in XRCC4−/− cells the fraction of error-prone repair events increased to 41% (P = 0.028), consistent with impaired precise ligation. The increase in error-prone religation of complementary ends in Ku80−/− cells was less pronounced, i.e. by 11% (Supplementary Figure S2B), and did not reach statistical significance.

Together, our data illustrate distinct roles of XRCC4 and Ku80 in NHEJ of I-SceI-induced DSBs with complementary and non-complementary ends. Loss of XRCC4 uniformly leads to a more severe repair defect than loss of Ku80.

Both XRCC4 and Ku80 are equally required for radiosensitivity and repair of IR-induced DSBs

After exposure to IR, DSB repair primarily relies on NHEJ. Accordingly, using constant-field gel electrophoresis, XRCC4 and Ku80 knock-out MEFs demonstrated a substantial DSB repair defect when compared to the respective wild-type strain (Figure 4B). Notably, loss of either gene resulted in a similar amount of unrepaired IR-induced DSBs, which is in contrast to the different I-SceI rejoining efficiencies seen in XRCC4+/− and Ku80+/− cells (Figures 2A and 3A). The observed impairment in the repair of radiation damage translated into a similar degree of cellular radiation hypersensitivity in both knock-out lines (Figure 4A).

DISCUSSION

XRCC4 promotes efficiency and fidelity of NHEJ

We characterize here the impact of murine XRCC4 deficiency on NHEJ of chromosomal DSBs on a molecular level. Upon loss of XRCC4, cells demonstrated a more than 20-fold decreased ability to rejoin I-SceI endonuclease-induced DSBs (Figure 2A) which is a much more pronounced difference than the effects reported for in vitro end-joining extrachromosomal NHEJ, or class switch recombination but similar to the effects upon V(D)J recombination (19,20,33–35). The importance of XRCC4 for the rejoining of enzymatic breaks extended to both, cohesive complementary DNA ends that are substrates for precise religation as well as non-complementarity ends that are typically rejoined along 1–2 nt of terminal A/T microhomology.

Others and we have found extended deletions and frequent usage of longer microhomologies in the absence of XRCC4 (this study, 19,24,35,36). Deletions are likely due to exonuclease activity in the absence of the XRCC4/LigIV complex. XRCC4 is further required for the recruitment and activation of the polymerase X family members Pol λ and Pol μ, which have emerged as the important polymerases in NHEJ (2,8,9,46). Reduced polymerase activity should result in unopposed exonuclease activity, which may lead to the long deletions observed in XRCC4+/− cells. Progressive end resection eventually exposes longer sequence homologies at the DNA ends, which may provide sufficient stability to
complete end-joining even without XRCC4/LigIV. It is tempting to speculate that DNA ligase III (LigIII) rejoins these ends. Longer microhomologies not only increase the stability of the junction but also better separate the free DNA ends. The gaps on each strand may be then recognized as individual SSBs, which are better substrates for LigIII than genuine DSBs (47). However, in our study only about 25% of junctions formed independently of any microhomologies (Figure 2D), thus additional mechanisms must exist for stabilization and synapsis of ends.

**Ku80 is dispensable for efficient NHEJ of site-directed DSBs**

Similar to the XRCC4−/− repair spectrum, Ku80−/− cells exhibited an increase in deletion size and a shift towards a mode of end-joining that relies on the use of longer microhomologies (3–5bp) (Figure 3C). This is in line with several previous reports (34,35,39,43,45,48,49). Interestingly, we could not detect a significantly reduced efficiency of end-joining in Ku80−/− compared to Ku80+/+ cells (Figure 3A, Supplementary Figure S3). Similar results were reported by Ma et al. for yeast (50) and Guirouilh-Barbat et al. (39) for hamster cell mutants despite notable differences in the chromosomal repair assays. The mild repair phenotype of Ku80−/− MEFs contrasts the severe rejoicing deficiency in XRCC4−/− cells, which mirrors the observed differences in embryonic viability seen with Ku80 versus XRCC4 knock-out mice (22,23). This raises the possibility that the chromosomal breaks created by the I-SceI endonuclease represent a better model of DSBs occurring during normal DNA metabolism and mouse development than biochemical or extrachromosomal end-joining systems.

Why is Ku80 not required for proficient end-joining? We cannot exclude the possibility that binding of the I-SceI enzyme and DNA cleavage may alter the chromatin structure sufficiently to attract other repair proteins, thereby alleviating the need for Ku to act as a signal amplifier and attractor of repair components. On the other hand, Ku is needed for the recruitment of XRCC4/LigIV (14, 16), yet we did not observe the same rejoicing defect in Ku80 cells as in XRCC4−/− cells. A more attractive explanation involves the repair pathway relying on LigIII, PARP-1, and XRCC1, which operates alternatively and independently of the DNA-PK-dependent pathway, as shown by the Salles and Iliakis laboratories (51–53). This alternative rejoicing activity was guided by the initial binding of PARP-1 to free DNA ends, which required the absence of Ku. Interestingly, the efficiency of this pathway could be significantly increased in LigIV-deficient cells by knock-down of Ku70 (50). This observation supports previous results showing that LigIV-deficient DT40 cells and even the embryonic lethality of LigIV−/− mice could be rescued by a simultaneous abrogation of Ku70 (54,55). Thus, repair in Ku80−/− MEFs may be channeled towards a LigIII-dependent pathway, which maintains rejoicing levels although at the expense of sequence loss at the break sites. In contrast, XRCC4-deficient cells express normal Ku80, which impairs access of PARP-1 to DNA. Hence, LigIII-mediated end-joining remains inefficient.

**Distinct roles for Ku80 and XRCC4 in the rejoining of I-SceI endonuclease versus IR-induced DSBs**

Why are the requirements for XRCC4 and Ku80 distinct for endonuclease- and IR-induced breaks? Enzymatic cleavage leaves clean termini while radiation-induced DNA ends are blocked by 5' dephosphorylated residues or 3' phosphoglycolates, which need to be removed prior to further processing. The resection of this type of ends requires the kinase activity of DNA-PK, and/or Artemis, and XRCC4/LigIV (11,48,56). Recruitment and proper activity of these proteins depend on Ku (7,14,16), thus explaining the high sensitivity of Ku80-deficient cells towards radiation but not enzymatic damage. In contrast, XRCC4 is needed for both types of breaks. An alternative but perhaps less likely possibility is that Ku80-independent end-joining mediates efficient repair of 1–2 DSBs that cannot cope with the large number of DSBs typically generated by IR.

In conclusion, our findings significantly advance our understanding of the roles of XRCC4 and Ku80 in chromosomal DSB repair. Both proteins are essential for the removal of IR-induced DSBs but only XRCC4 is required for efficient rejoicing of enzymatic breaks, which may resemble spontaneous DSBs arising during normal DNA metabolism. Our results can thus help us appreciate why the XRCC4−/− but not the Ku80−/− phenotype is lethal in mice.

While this manuscript was under revision Lopez and colleagues published a complementary work (57) that lead to similar conclusions, i.e. that only loss of XRCC4 but not of Ku80 reduced the efficiency of the repair of I-SceI induced breaks

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

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**Conflict of interest statement.** None declared.

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