# Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks

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### ABSTRACT

In mammalian cells, the main pathway for DNA double-strand breaks (DSBs) repair is classical non-homologous end joining (C-NHEJ). An alternative or back-up NHEJ (B-NHEJ) pathway has emerged which operates preferentially under C-NHEJ defective conditions. Although B-NHEJ appears particularly relevant to genomic instability associated with cancer, its components and regulation are still largely unknown. To get insights into this pathway, we have knocked-down Ku, the main contributor to C-NHEJ. Thus, models of human cell lines have been engineered in which the expression of Ku70/80 heterodimer can be significantly lowered by the conditional induction of a shRNA against Ku70. On Ku reduction in cells, resulting NHEJ competent protein extracts showed a shift from Cto B-NHEJ that could be reversed by addition of purified Ku protein. Using a cellular fractionation protocol after treatment with a strong DSBs inducer followed by western blotting or immunostaining, we established that, among C-NHEJ factors, Ku is the main counteracting factor against mobilization of PARP1 and the MRN complex to damaged chromatin. In addition, Ku limits PAR synthesis and single-stranded DNA production in response to DSBs. These data support the involvement of PARP1 and the MRN proteins in the B-NHEJ route for the repair of DNA DSBs.

### INTRODUCTION

Double-strand break (DSB) is toxic DNA damage that, if improperly repaired, can lead to cell death or cancer following genomic rearrangement (1). DSBs are formed in response to endogenous cellular processes such as V(D)J recombination, Class Switch Recombination (CSR) and oxidative metabolism in addition to genotoxic agents such as ionizing radiation, radiomimetic compounds and topoisomerase inhibitors. In mammalian cells, the main pathway for DSB repair is canonical non-homologous end joining (thereafter named C-NHEJ), which throughout the cell cycle ligates the two DNA ends together with minimal end processing (2–4).

C-NHEJ is a multi-step process involving several essential factors (5,6). The prerequisite event for all the subsequent steps is the binding of Ku70/Ku80 heterodimer to DNA ends (7). In the most recent model drawn from live cell imaging following nuclear laser micro-irradiation experiments, the other core components of the reaction are then independently recruited to Ku-bound DSB (8). These include the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Cernunnos-XLF (Cer-XLF) and the XRCC4/DNA Ligase IV (LIG4) complex which is preassembled by a tight association between the two partners (9). Multiple interactions then take place

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among these factors resulting in stable assembly of the NHEJ machinery. As a result the NHEJ complex associates more tightly with damaged sites and becomes resistant to biochemical extraction from the damaged chromatin, at least during the repair time (10-12). DNA-PK holoenzyme (Ku/DNA-PKcs) carries out recognition, protection and bridging activities on the DNA-ends in addition to a serine/threonine protein kinase activity (13). DNA-PK conformational change mediated by autophosphorylation is necessary for activation of end-processing enzymes such as the ARTEMIS nuclease (14). DNA-PK may also function outside DNA repair through phosphorylation of other substrates (15,16). Ligation requires the concerted action of LIG4, XRCC4 and Cer-XLF. the latter promoting re-adenylation of LIG4 (17). The ligation complex also has a role upstream the ligation reaction since it stimulates processing of DNA ends (18,19).

Recently, evidence has accumulated in yeast as well as in mammalian cells of an alternative or backup NHEJ route (thereafter named B-NHEJ) which accounts for residual end-joining of DSB in cells deficient in components of C-NHEJ (20-23). B-NHEJ may also operate at telomeres in telomerase-deficient mouse cells (24) or following a defect of Ku or DNA-PKcs (25.26). This alternative pathway may be particularly relevant to genomic instability associated with cancer. For example, frequent translocations lead to a high level of lymphomagenesis and other cancers in C-NHEJ deficient animal models (27,28). In addition, chromosomal translocations like those at the origin of leukemia are mediated by a rejoining pathway which is mostly Ku- and XRCC4/ LIG4-independent (29-32). Thus, deciphering the components and the mechanisms of these pathways is an important step in the understanding of tumorigenesis

Established features of the B-NHEJ pathway include : (i) kinetics of DSB repair appears slower than C-NHEJ (33,34) and enhanced in G2 (35); (ii) it is repressed by Ku under normal conditions (29,34,36–40); (iii) it relies preferentially on resection of DNA ends and ends annealing driven by microhomology (MH) >4 bp for intrachromosomal substrates (36,37,41,42), V(D)J junctions (43) or CSR joins (30,40), although this feature has been questioned in some reports (44).

Studies in cells have implicated members of the MRN complex in B-NHEJ (45–51), together with PARP1 and XRCC1/DNA Ligase III (LIG3) proteins, otherwise acting in base excision repair (BER) (44,52–55). Our group and others have characterized some features of B-NHEJ using biochemical assays with cell extracts. It has been shown *in vitro* that Ku competes with PARP1 DNA end-binding, that PARP1 can perform a synapsis activity owing to short homology at the DNA ends and that PARP1 activity is required for a subsequent XRCC1/LIG3 joining step favored by MH (34,52,53,56).

One missing link between these genetic and biochemical data is the characterization in human cells of the nuclear mobilization of candidate B-NHEJ proteins in response to DSB at early time points following damage infliction. Since C-NHEJ represses other DSB repair mechanisms possibly through Ku binding to DNA ends, little chance exists in normal human cells for the isolation of B-NHEJ proteins at DNA breaks. In addition, Ku is an essential protein in humans probably through telomere stabilization (57) and no stable human Ku knock-out cell line exists, although conditional genetic ablation of Ku80 has been described (39,57). Here, we have engineered models of human cell lines in which the expression of Ku can be significantly lowered by the conditional induction of a shRNA against Ku70. These cells offer the opportunity to assess the function of Ku in antagonizing other DNA end-binding activities and to study the mobilization of candidate repair proteins other than C-NHEJ factors after DSB infliction.

We report that after Ku reduction in cells, NHEJ competent protein extracts obtained from these cells showed a shift from C- to B-NHEJ that could be reversed by addition of purified Ku protein. Using a cellular fractionation protocol after treatment with a strong DSB inducer, we found that mobilization to damaged chromatin of PARP1 and the MRN complex increases preferentially upon Ku-deficiency compared to other conditions of NHEJ deficiency. Enhanced recruitment of these proteins to damaged chromatin was correlated with an increase in poly(ADP-ribose) (PAR) synthesis and single-stranded DNA (ssDNA) production. Our data support the involvement of these proteins in the B-NHEJ pathway for the repair of DNA DSBs.

## MATERIALS AND METHODS

#### Cell lines and cell culture

Cells were grown in a 5%  $CO_2$  humidified incubator at 37°C. All culture media and antibiotics were from Invitrogen. Media were supplemented with 10% fetal calf serum, 125 U/ml penicillin and 125 µg/ml streptomvcin. HT1080 human fibrosarcoma cells and MRC5-SV immortalized human non-tumoral fibroblasts were grown in DMEM. MRC5-SV cells that stably express an shRNA against LIG4 or a control shRNA were maintained in D-MEM supplemented with 0.1 mg/ ml hygromycin B (9). DNA-PKcs-deficient and -complemented cell lines [Fus9 and Fus1, respectively (58) gift from C. Kirchgessner, Standford University School of Medecine, CA, USA] were maintained in D-MEM-F12 1/1 medium.

#### Expression vectors, cell transfection and transduction

Both pLV-tTR-KRAB-Red and pLVTHM vectors were obtained from Tronolab (59). pLV-tTR-KRAB-Red is a lentiviral vector encoding the transcriptional repressor tTR-KRAB fused to the DsRed fluorescent protein. pLVTHM is a lentiviral vector allowing conditional expression of an shRNA of interest under the control of the H1 promoter and the tetracyclin operator/ repressor system (TetO/TetR). The pLVTHM-shKu70 vector allowing conditional expression of an shRNA against Ku70 was obtained by inserting a duplex oligonucleotide (sh70-1: 5'-CGC GTC CCC GAG TGA AGA TGA GTT GAC ATT CAA GAG ATG TCA ACT CAT CTT CAC TCT TTT TGG AAA T-3'; sh70-2: 5'-CGA

TTT CCA AAA AGA GTG AAG ATG AGT TGA CAT CTC TTG AAT GTC AAC TCA TCT TCA CTC GGG GA-3') between MluI and ClaI restriction sites in the pLVTHM plasmid. The targeted sequence corresponds to nucleotides 156-174 of the human Ku70 coding sequence. Transfection of HEK-293 T cells (kindly by Genethon, Evry, provided France) with pLV-tTR-KRAB-Red or pLVTHM-shKu70, and preparation of high titer lentiviruses pseudotyped with VSV-G protein have been performed as previously described (60). For the transduction step, fifty thousand HT1080 cells or MRC5 cells were plated on 35-mm dishes 24h prior to co-transduction with the two different viral vectors at an MOI of 10:1. Individual clones from the transduced cell population were then isolated and selected for their capacity to downregulate Ku70 expression under treatment by the tetracyclin analog doxycyclin. The data presented were obtained on one clone of each cell type.

For construction of the sh-resistant Ku70 expressing vector, the pcDNA3-Neo vector (Invitrogen) was modified so as to contain a FLAG tag encoding sequence between HindIII and BamHI restriction sites (pcDNA3-Neo-FLAG). The human Ku70 cDNA was modified by PCR in order to introduce silent mutations to obtain an shRNA-resistant version of the corresponding Ku70 mRNA. This shRNA-resistant Ku70 cDNA was then inserted into the BamHI and NotI sites of the pcDNA3-Neo-FLAG in frame with the FLAG tag encoding sequence.

#### Chemicals and cell treatment

Calicheamicin- $\gamma$ 1 (Cali), a generous gift from PR Hamann (Wyeth Research, Pearl River, NY, USA), was dissolved at 4.4 mM in ethanol and stored at  $-80^{\circ}$ C. DPQ (3,4-dihydro-5[4-(1-piperindinyl)butoxy]-1(2H)-

isoquinoline, Sigma) and NU7026 (Calbiochem) were dissolved in DMSO (10 mM stock solution) and stored at  $-20^{\circ}$ C. Small aliquots of these stock solutions were used once. Doxycyclin (Sigma) was stored at  $-20^{\circ}$ C as a 10 mg/ml stock solution in sterile water. Methyl methane sulfonate (MMS) was purchased from Sigma.

When necessary, cells were incubated in the presence of doxycyclin  $(4 \mu g/ml)$  in the growth medium for the time required and the medium was renewed every 3 or 4 days. For exposure to DNA-damaging treatment, cells were exposed to freshly diluted Cali (44 nM) or MMS (10 mM) in medium for 1 h unless other indication, at  $37^{\circ}$ C in culture dishes and then harvested at the indicated time points. When necessary, DPQ or NU7026 at concentrations as indicated were added to the culture medium for 1 h and maintained during treatment with Cali.

#### Antibodies

The antibodies used were: mouse monoclonal anti-DNA-PKcs (18-2), anti-Ku80 (S10B1), anti-Ku70 (N3H10), anti-Ku (p70/p80) (clone 162, Thermo Fisher Scientific), anti-XRCC1 (Neomarkers), anti-LIG3, anti-MRE11, anti-NBS1 (BD transduction laboratories), anti-Rad50 (13B3, GeneTex), anti-BrdU (Roche), anti- $\beta$  Actin (AC-15, Ambion), anti-PARP1 (4C10-5, BD

Pharmingen), anti-PARP1 (C2-10, Trevigen), anti-PAR (Trevigen), anti- $\alpha$  tubulin (Sigma), anti-SAF-B (6F7, Abcam), anti- $\gamma$ H2AX (JBW301, Upstate Biotechnology), anti-RPAp34 (9H8, gift of MS Wold, Carver College of Medicine, University of Iowa, USA) and rabbit polyclonal: anti-XRCC4 (produced in our laboratory), anti-DNA LIG4 (Serotec), anti-phospho-Ser2056-DNA-PKcs (gift of DJ Chen, UTSMC, Dallas, TX, USA), anti-H2AX (GeneTex). Goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxydase were from Jackson Immunoresearch Laboratories, Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG (1:600) were from Molecular Probes, Invitrogen.

#### End-joining extracts and protein purification

Cell extract preparation and Ku protein purification were carried out as previously described (26).

#### End-joining assay

When necessary, extracts were preincubated with NU7026 (Sigma-Aldrich), or purified Ku protein as indicated for 10 min at  $4^{\circ}$ C. Pretreated or mock-treated extracts (40 µg) were incubated for 2h at 25°C in 10 µl reaction mixture containing 5 ng EcorRI-linearized pBluescript-KS-II(-) plasmid in EJ buffer (50 mM Triethanolamine pH 8.0, 0.5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mg/ ml BSA, 60 mM potassium acetate) with 1 mM ATP added at last to initiate the reaction. Samples were then treated with 100 µg/ml RNAse A for 10 min at 37°C and deproteinized. DNA ligation products were separated in 0.7% agarose gels together with GeneRuler<sup>TM</sup> DNA ladder mix (0.5-10 kb, Fermentas) in one lane and stained with SYBR-Green (Invitrogen). Fluorescence was detected and analyzed on a Typhoon fluorimager (Molecular Dynamics). Quantitative analysis of the gel was performed with the ImageJ software (version 1.4).

#### **Biochemical fractionation and immunoblotting**

After drug exposure, cells were washed with phosphatebuffered saline (PBS) and harvested. Pellets of  $\sim 5 \times 10^6$ cells were fractionated as reported (61) with minor modifications. Briefly, cells were first resuspended for 5 min on ice in 200 µl of fractionation buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 0.05% Nonidet P-40 (NP40) and supplemented with the Halt protease and phosphatase inhibitor cocktail (Pierce). Following centrifugation at  $1000 \times g$  for 5 min, the supernatant was collected (fraction I), and pellets were incubated in 200 µl of the same buffer supplemented with 100 µg/ml RNaseA (Sigma) for 10 min at 20°C. The supernatant was collected as before (fraction II), and the nuclear pellets were further extracted for 40 min on ice with 200 µl of fractionation buffer containing 0.5% Nonidet P-40. The extracts were clarified by centrifugation at 16000 g for 15 min (fraction III). The pellets were resuspended in 200 µl extraction buffer supplemented with 1% Triton X-100 and 0.45 M Nacl and sonicated (Vibracel, Bioblock Scientific).

Whole-cell extracts (WCE) were obtained by direct lysis in extraction buffer (50 mM HEPES, pH 7.5, 450 mM NaCl, 1 mM EDTA, 1% Triton) followed by sonication. Protein concentration was determined by the Bradford assay (BioRad).

Aliquots of soluble or chromatin fractions I–IV, derived from equivalent cell numbers for each culture conditions, or WCE samples with the same amount of proteins were added to loading buffer, boiled and loaded on SDS– PAGE. After migration, proteins were blotted to PVDF membrane (Millipore). Immunoblots were visualized by enhanced chemiluminescence (Yelen).

# In situ detergent extraction and immunofluorescence

Cells incubated in the presence or absence of doxycyclin for 8 days and plated onto coverslips were mock-treated or treated with Calicheamicin at the dose indicated. After 45 min of drug exposure, cells were washed in PBS then fixed in 4% paraformaldehyde for 20 min at RT and permeabilized by a 5-min incubation with 0.2% Triton X-100 in PBS at RT. After each step, the coverslips were rinsed three times with PBS. Coverslips were blocked with 3% BSA and RNAse A (100 µg/ml) in PBS for 60 min at RT. Then cells were incubated with the appropriate primary antibodies in PBS, 2% BSA for 2h at RT. After extensive washing with PBS, antibody binding was detected by incubation with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG (1:600) for 1 h at RT. DNA was stained with 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma) for 5 min at RT. Coverslips were mounted in Dako mounting medium. Images were taken with a (DM5000) microscope equipped Leica with а CoolSnapES camera. Images were processed for publication using the Adobe PhotoShop 7.0 software program.

For in situ detergent extraction experiments, cells were grown on collagen (PARP1 immunostaining) or poly-Llysine (Rad50 immunostaining) coated glass coverslips, mock-treated or treated with Calicheamicin at the dose indicated. Pre-extraction was carried out by incubating the coverslips in extraction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 300 mM sucrose, 2 mM MgCl<sub>2</sub>) containing 0.1% Triton X-100 on ice for 5 min for Cali-treated cells and 8 min for mock-treated cells. Non pre-extracted control cells were incubated in the same buffer without detergent. Immunolabeling with anti-PARP antibodies (1:1000)and anti-RAD50 antibodies (1:500) was performed as described above.

# In situ ssDNA detection

Visualization of ssDNA *in situ* was performed by following the protocol of Radershall *et al.* (62) with minor modifications. Briefly, cells incubated with doxycyclin for 8 days were labeled with  $20 \,\mu$ M 5' bromo 2' deoxyuridine (BrdU, Sigma) for 6 days before immunofluorescent staining while proliferating control cells were incubated with BrdU for 48 h. Cells plated on glass coverslips were treated with Calicheamicin for 45 min, post-incubated in fresh medium, then washed with PBS and processed for immunofluorescent staining at different times posttreatment. After fixation in ice-cold methanol for 10 min at  $-20^{\circ}$ C and blocking with 3% BSA in PBS for 1 h, cells were immunolabeled with monoclonal anti-BrdU antibody (1:50) for 1 h at room temperature followed by incubation with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG (1:600) for 1 h at room temperature. To check for BrdU incorporation into nuclear DNA, a denaturation step was performed by incubating coverslips in 2N HCl for 10 min. After extensive washing with PBS, cells were subjected to BrdU immunostaining as described above.

### Analysis of poly(ADP-ribose) (PAR) synthesis

Cells seeded onto coverslips were mock treated or treated with 40  $\mu$ M DPQ for 1 h at 37°C, then 40 nM Calicheamicin was added to the medium for 7 min. Cells were washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature followed by a 5-min incubation with 0.2% Triton X-100 in PBS. Cells were then immunolabeled using anti-PAR antibodies (1:1000, 2 h, RT) and Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse secondary antibody. As a control, cells incubated in the presence or absence of DPQ (40  $\mu$ M, 1 h) were treated with 1 mM H2O2 for 15 min at 37°C. Cells were washed in PBS, fixed and permeabilized, then immunolabeled with anti-PAR antibodies (1:1000) as described above.

# RESULTS

# Cell construction and characterization

Ku is essential in human cells (57.63). To get rid of Ku binding at DSBs and allow access to candidate repair proteins for alternative DSB repair pathways, we established a human cell line in which Ku70 expression could be conditionally knocked-down. Human fibrosarcoma HT1080 cells were doubly transduced with lentiviral vectors so as to express a shRNA against Ku70 under the control of a tetracyclin repressor. Cell clones were subsequently isolated and showed a very efficient knock-down of Ku70 expression under the conditional induction of anti-Ku70 shRNA with doxycyclin (Figure 1A). As expected from the well-known reciprocal stabilization of both Ku subunits, Ku80 degradation paralleled Ku70 fade-out (Figure 1A). After 8 days induction,  $\sim 95\%$  reduction of Ku expression level was achieved (Figure 1A and unpublished data). Conversely, all other C-NHEJ components, as well as other DNA repair proteins were not significantly affected by the decrease of Ku70/Ku80 expression level. Consistent with the essential function of Ku in human somatic cells (57,63), cell proliferation was progressively slowed down and stopped under prolonged Ku reduction, and cells went on to die after 12 days (unpublished data).

Then NHEJ competent extracts obtained from Ku-proficient or deficient cells were assayed for their end-joining capacity on linear plasmid (Figure 1B). Notably, both extracts showed roughly identical activity. However activity of Ku-proficient extract was sensitive to a specific DNA-PK inhibitor while that of Ku-deficient extract was partially resistant (~40% mean remaining ligation activity in three experiments). In addition, anti-XRCC4 antibodies completely inhibited end-joining in Ku-proficient extracts but only partially in Ku-deficient



**Figure 1.** Effect of Ku extinction on the expression and mobilization to damaged chromatin of representative repair proteins and on NHEJ activity *in vitro*. (A) WCEs of HT1080 shKu70 cells treated with doxycyclin for the indicated time were denatured and separated on 10% SDS–PAGE gel followed by electrotransfer on membrane. The membranes were blotted with the antibodies as indicated. (B) End-joining assay catalyzed under standard reaction conditions with extracts from Ku proficient or deficient cells (treated with doxycylin for 7 days), in the presence or not of DNA-PK specific inhibitor NU7026. DNA ligation products were separated by agarose gel electrophoresis followed by SYBR-Green staining. Ligation efficiency (% of plasmid multimers versus monomer) was 12.4 and 15.1 for lanes 1 and 3 (without NU7026) and 0 and 5.5 for lanes 2 and 4 (with NU7026), respectively. (C) End-joining assay catalyzed under standard reaction conditions with extracts from Ku proficient cells (treated with doxycylin for 7 days), in the presence or not of the indicated amount of purified Ku protein. DNA ligation products were separated by agarose gel electrophoresis followed by SYBR-Green staining. Ligation efficiency (% of multimers versus monomer) was 13.7, 12.9, 16.6, 16.2 and 15.5 for lanes 1, 3, 5, 7 and 9 (without NU7026) and 0, 6.0, 2.6, 1.7 and 0.9 for lanes 2, 4, 6, 8 and 10 (with NU7026), respectively. (D) HT1080 shKu70 cells were treated (T) or not (NT) with calicheamicin (Cali), collected and fractionated as described in the 'Materials and Methods' section, leading to fractions I to IV. Protein samples were denatured and separated on SDS–PAGE gels 10% for standard separation or 15% for  $\gamma$ H2AX isolation, followed by electrotransfer and blotting as indicated. (E) HT1080 shKu70 cells were grown for 12 days in the presence or not of doxycyclin, then treated with Cali, fractionated and analyzed as in (D).

extracts (data not shown). Together, these results indicate a shift from C- to B-NHEJ upon Ku reduction in cells, as we previously observed after Ku depletion from extracts (26). Since co-depletion of some component in addition to Ku could have occurred in cells, we added back purified human Ku dimer to the extracts. Sensitivity to the DNA-PK inhibitor was progressively restored upon addition of increasing amount of Ku to extracts from the Ku-depleted cells, indicating a reverse shift from Bto C-NHEJ (Figure 1C, <6% remaining ligation activity for the highest amount of Ku added). Therefore, Ku was a major inhibitor of alternative end-joining assessed in cell extracts.

# Cell fractionation after DSB production in chromatin under Ku deficiency

We have adapted a cellular fractionation procedure to reveal chromatin association of repair proteins at early time following DSB induction (10,61). Fractionation procedure is based on successive detergent extractions (fraction I–IV, from cytoplasmic to chromatin fraction, respectively, characterized in Supplementary Figure S1), aimed at removing loosely bound proteins. This protocol or a derivative from our laboratory has allowed visualization of the association of repair or signaling proteins with sites of DSB in chromatin (10,11,61,64,65).

For production of DSB, non-induced HT1080 shKu70 cells were treated with Calicheamicin-y1 (Cali), yielding a 1:3 ratio of DNA DSB to single-strand breaks (SSBs) in vivo, compared to a <1:20 ratio for ionizing radiation (66). In addition, it has been estimated that under experimental conditions similar to ours, Cali produced at least 400 DSBs/nM per cell (66). Then the mobilization of three groups of proteins were compared following DSB production; the C-NHEJ pathway (Ku70, DNA-PKcs, XRCC4, LIG4); the BER pathway (PARP1, XRCC1, LIG3); or the MRN complex (MRE11 and NBS1). The four fractions obtained after extraction of equivalent number of cells under each culture conditions were analyzed by immunoblot following SDS-PAGE (Figure 1D). As expected, the majority of C-NHEJ proteins were released in the early extracted fractions (I and II) in non-treated cells. In contrast, the C-NHEJ proteins mainly shifted to the extraction-resistant fractions (III and IV) in cells treated with Cali for 1h prior fractionation; these fractions also contained the phosphorylated form of H2AX as DSB marker. In addition XRCC4 migrated as a retarded band corresponding to DNA-PK-dependent phosphorylation, as previously published (10). Although PARP1 and XRCC1/LIG3 were also confined in the extractable fractions I and II in non-treated cells, they behaved differently from C-NHEJ proteins in treated cells, i.e. they mainly remained extractable and were only partially recruited to chromatin (Figure 1D). Although the Cali treatment results in a concomitant production of base damage and SSBs, this low retention may be explained non-exclusively by the essentially transient attachment of PARP1 at the damage site (67) and/or improper extraction conditions for SSB-binding proteins. Finally, no mobilization of MRE11 and NBS1 to the chromatin fraction was detected following Cali treatment under these conditions (Figure 1D).

We then compared the distribution in the four fractions of the three groups of proteins after Cali treatment of HT1080 shKu70 under non-induced or induced conditions for the expression of Ku70 shRNA (Figure 1E). Same amount of phosphorylated H2AX was present in the insoluble fraction IV under both conditions (Figure 1E). Upon shKu70 expression, the  $\sim 5\%$  remaining amount of Ku was mostly present in fraction IV. Under normal Ku expression, DNA-PKcs was present in fractions III and IV while under limited Ku expression, it rather shifted to the more soluble fractions I and II, indicating a poorer retention in the damaged chromatin. Under limited Ku expression, XRCC4 was still present in the chromatin fraction IV and the LIG4 profile appeared unchanged (Figure 1E). However, on Ku reduction, XRCC4 appeared broadly less phosphorylated over the four fractions as compared with the exclusive hyperphosphorylated form observed under normal conditions of Ku expression. Regarding PARP1 and MRN proteins, a clear enrichment was observed in fractions III and/or IV under limited Ku expression (Figure 1E), as also found to a lesser extent for XRCC1/LIG3 proteins.

Hypo-phosphorylation of XRCC4 suggested a defect in DNA-PKcs activity since under these conditions this protein was essentially phosphorylated by DNA-PKcs

[(10) and Supplementary Figure S2]. Thus we checked the phosphorylation of the p34 subunit of RPA, another DNA-PKcs substrate after DSB production, as shown in Supplementary Figure S2. Figure 1E shows a shift in the distribution of RPAp34 forms to the less phosphorylated ones upon limiting Ku expression, supporting the hypothesis of a defect in DNA-PKcs activation.

To further test this hypothesis, we used another cellular model of Ku deficiency. The system for conditional induction of Ku70 shRNA was introduced into MRC5 cells (SV40 transformed human lung fibroblasts) and led to very efficient knock-down of Ku70 expression after 8 days in the presence of doxycylin (Figure 2A and C). Notably, after Cali treatment, Ku deficiency led to accumulation of PARP1, XRCC1/LIG3 and proteins of the MRN complex in the less extractable fractions III and IV, while only minor change occurred in the XRCC4 and LIG4 profile, except a defect in XRCC4 phosphorylation along with a concomitant defect in RPAp34 phosphorylation (Figure 2B).

We then used another technique to validate these results. After Cali treatment under conditions of Ku extinction in MRC5 cells (Figure 2C), immunofluorescence detection revealed a strong reduction in the amount of Ku present in the chromatin fraction compared to nondepleted cells (Figure 2D). In contrast, and as observed by western-blotting, PARP1 was much more detected in the damaged chromatin of Ku-depleted cells after treatment with Cali than in the damaged chromatin of Ku-proficient cells (Figure 2E). Similarly, the damaged chromatin was clearly enriched in Rad50 upon Ku reduction in cells (Figure 2F).

# Ku is the main inhibitor of PARP1 and MRN recruitment to chromatin containing DSBs

Ku deficiency increased recruitment of PARP1, MRN and to a lesser extent XRCC1/LIG3 proteins to less soluble fractions after Cali treatment. This result suggests the release of a Ku-mediated impairment of the direct or indirect binding of these proteins to DSBs. To address this point, we compared protein mobilization under normal or Ku-deficient conditions after cell treatment with a high dose of MMS which produces SSBs as intermediate lesions during BER repair of methylated DNA bases. Without doxycyclin and as expected, this treatment failed to recruit C-NHEJ proteins to insoluble fractions (Supplementary Figure S3). We previously observed this failure, unless un-repaired SSBs due to MMS treatment were converted into DSBs by PARP inhibition (12). Likewise, MRN proteins remained soluble after MMS treatment. In contrast, there was a strong displacement of PARP1, XRCC1 and LIG3 to fraction IV following MMS treatment. However, no benefit of Ku reduction was observed on the mobilization of BER or MRN proteins to SSBs (Supplementary Figure S3), contrarily to what was found after treatment with Calicheamicin (Figures 1E, 2B, E and F). Hence, DSBs appear to be mostly responsible for the mobilization of these proteins to damaged chromatin upon Ku reduction.



**Figure 2.** Effect of Ku extinction on the expression and mobilization to damaged chromatin of representative repair proteins. (A) WCEs of MRC5 shKu70 cells treated with doxycyclin for the indicated time were denatured and separated on 10% SDS–PAGE gel followed by electrotransfer on membrane. The membranes were blotted with the antibodies as indicated. (B) MRC5 shKu70 cells were grown for 9 days in the presence or not of doxycyclin, then treated with Cali, fractionated as described in the 'Materials and Methods' section, leading to fractions I–IV. Protein samples were denatured and separated on SDS–PAGE gel, followed by electrotransfer and blotting as indicated. (C) MRC5 shKu70 incubated or not with doxycyclin for 8 days were fixed, permeabilized and immunostained with anti-Ku70/Ku80 heterodimer antibodies followed by incubation with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with DAPI. (D) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated or treated with 10 nM Calicheamicin (Cali) for 45 min at 37°C in medium. Cells were permeabilized in buffer containing 0.1% Triton X-100 prior to fixation and immunostaining with anti-Ku70/Ku80 heterodimer antibodies and Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse antibodies. DNA was stained with DAPI. (E and F) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated with 10 nM Cali for 45 min at 37°C in medium. Cells were pre-extracted in buffer containing 0.1% Triton X-100 prior to fixation and immunostaining with anti-Ku70/Ku80 heterodimer antibodies and Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgC on the fixed or not with doxycyclin for 8 days were mock-treated with DAPI. (E and F) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated anti-mouse antibodies. DNA was stained with DAPI. (E and F) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated anti-mouse antibodies. DNA was stained with DAPI. (E and F) MRC5 shKu70 incubated or not with dox-Fluor<sup>®</sup> 594-conju

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Figure 2. Continued.

An important issue was to control that the doxycyclin treatment *per se* had no side effect on the recruitment of repair proteins. Therefore, HT1080 shKu70 cells were complemented with a construction expressing a shRNA-resistant FLAG-tagged Ku70 mutant. As expected, these cells still expressed FLAG-Ku70 12 days after treatment with doxycyclin whereas expression of the endogenous protein was strongly decreased (Figure 3A) and this led

an efficient growth defect complementation (unpublished results). Protein recruitment after Cali treatment was then compared in these cells treated or not with doxycyclin for 12 days (Figure 3B). Upon Cali treatment in doxycyclin-treated cells, FLAG-Ku70 was present in fractions III and IV in concentration well over the trace amount of the remaining endogenous Ku70 protein recruited. Although under these conditions, the total

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Figure 3. Effect of Ku restoration and LIG4-deficiency on the mobilization to damaged chromatin of representative repair proteins. (A) WCEs of HT1080 shKu70/Ku70shR cells treated with doxycyclin for the indicated time were denatured and separated on 10% SDS–PAGE gel, followed by electrotransfer and blotting as indicated. Asterisk indicates FLAG-Ku70. (B) HT1080 shKu70/Ku70shR cells were grown for 12 days in the presence or not of doxycyclin, then treated with Cali, collected and fractionated as described in the 'Materials and Methods' section, leading to fractions I–IV. Protein samples were denatured and separated on 10% SDS–PAGE gel, followed by electrotransfer and blotting as indicated. Asterisk indicates FLAG-Ku70. (C) Western blotting on the WCEs of the MRC5 cells expressing or not shLIG4. (D) MRC5 cells expressing or not shLIG4 were treated with Cali, fractionated and analyzed as in (B).

amount of Ku in these fractions was lower than in the damaged doxycyclin non-treated cells, recruitment and activation of DNA-PKcs was sufficient to phosphorylate XRCC4 as efficiently as in doxycyclin non-treated cells (Figure 3B). Strikingly, a similar minor mobilization of BER and MRN proteins to the insoluble fractions III and IV was observed in the damaged cells treated or not with doxycyclin. These results clearly rule out the hypothesis that a secondary effect of doxycyclin might promote the mobilization of these proteins to the damaged chromatin. In addition, the fact that the maintenance of Ku expression prevented an enhanced recruitment of BER and MRN proteins to chromatin damaged with DSB suggests that Ku is a main inhibitor of the recruitment of these proteins.

Ku binding to DNA-ends initiates the C-NHEJ pathway which is thus strongly compromised in the absence of the Ku heterodimer (7). The repair defect could somehow mediate the change in the balance between NHEJ and the other repair proteins in the less extractable fractions after Cali treatment. To address this hypothesis, the same extraction after DSB infliction was performed under NHEJ deficiency mediated by silencing the other NHEJ factor LIG4. Stable expression of shLIG4 led to a strong reduction in LIG4 expression as published (9) (Figure 3C). As shown in Figure 3D, LIG4 failed to accumulate in fractions III and IV in cells expressing shLIG4. LIG4 deficiency also impaired XRCC4 accumulation in these fractions, as expected from our published data (9,12). However, Ku was present on chromatin in LIG4 proficient or deficient cells and no extra accumulation of PARP1, LIG3, MRE11 or NBS1 proteins was noticed in fractions III and IV under these LIG4 deficiency conditions (Figure 3B). This implies that Ku decreased expression *per se* and not the resulting NHEJ defect was responsible for the extra-mobilization of PARP1 and MRN proteins to the chromatin damaged with DSBs observed after Cali treatment.

Since Ku is the regulatory subunit of the DNA-PK complex, it was also possible that the kinase activity of the complex be the main contributor of the exclusion of BER and MRN proteins from DSBs.

To test this possibility, we first analyzed fractionation after Cali treatment of the human DNA-PKcs-deficient glioblastoma cell line (Fus9) compared to its *DNA-PKcs* complemented counterpart (Fus1) (Figure 4A). Calicheamicin induced a similar recruitment of Ku to damaged chromatin in both DNA-PKcs proficient or deficient cells. Although DNA-PKcs was not expressed in FUS9 cells, PARP1 and NBS1 remained mostly soluble, to the same extent as in FUS1 cells. Thus, this indicates that the over-mobilization to damaged chromatin of PARP1 and MRN proteins that was observed upon Ku

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**Figure 4.** Effect of DNA-PKcs deficiency or inhibition of DNA-PK on the mobilization to damaged chromatin of representative repair proteins. (A) DNA-PKcs deficient (Fus9) and DNA-PKcs complemented (Fus1) glioblastoma cell lines were treated with Cali. collected and fractionated as described in the 'Materials and Methods' section, leading to fractions I to IV. Protein samples were denatured and separated on 10% SDS–PAGE gel, followed by electrotransfer and blotting as indicated. (B) HT1080 shKu70 cells were grown for 12 days in the presence or not of doxycyclin, then treated with Cali in the presence of increasing concentrations of the DNA-PKcs inhibitor NU7026 as indicated, fractionated and analyzed as in (A), except that only pooled fractions III and IV were loaded.

under-expression was not associated with DNA-PKcs inactivation.

To evaluate a possible contribution of DNA-PKcs that could have escaped the previous analysis, we checked the effect of the selective DNA-PKcs inhibitor NU7026 on the recruitment of PARP1, LIG3 and MRN proteins to the damaged chromatin (as a pool of fractions III and IV) in doxycyclin-untreated cells as compared to the optimum recruitment in doxycyclin-treated cells (Figure 4B). Increasing doses of NU7026 progressively inhibited DNA-PKcs activation as shown by the inhibition of DNA-PKcs auto-phosphorylation on S2056 (68,69), as well as by inhibition of phosphorylation of DNA-PKcs substrates (Supplementary Figure S2). Interestingly, this inhibition paralleled a progressive increase in the recovery of PARP1, LIG3 and MRE11 in the damaged chromatin, although the amount of Ku recruited remained constant. However, for the highest dose of inhibitor, the concentration of these proteins in the chromatin fraction remained far below that observed upon Ku reduction, although the level of DNA-PKcs phosphorylation on S2056 was similar under both conditions (Figure 4B). This indicates that although Ku is the main inhibitor of PARP1, LIG3 and MRN protein recruitment to DSB, DNA-PKcs activity also contributes slightly to this inhibition.

# Functional consequences of PARP and MRN recruitment at DSBs

PARP1 is the major poly(ADP-ribose) (PAR) synthetizer in cells in response to DNA SSBs (70). Indeed, a strong PAR pan-nuclear signal was observed in MRC5 shortly after treatment with  $H_2O_2$  but was hardly detected when cells were preincubated with the PARP inhibitor DPQ (Figure 5A). Then, we checked PAR production after treatment with Cali in Ku-proficient and -deficient cells. Strikingly, Ku-deficient cells showed the strongest nuclear PAR signal that was DPQ-sensitive (Figure 5B and Supplementary Figure S4). In addition, we found that PARP1 recruited to DSB upon Ku reduction impacted in turn the residual DNA-PKcs activity: PARP inhibition combined with Cali-treatment in cells under-expressing Ku led to an increased phosphorylation of XRCC4 and RPAp34 DNA-PKcs substrates (Supplementary Figure S5).

Since the MRN complex comprising MRE11 nuclease was over-recruited in damaged chromatin after DSBs production in the absence of Ku, we aimed at estimating the amount of ssDNA under these conditions. The thymidine analog BrdU was incorporated into DNA during cell growth, as detected after *in situ* DNA denaturation and labeling with a specific antibody (Figure 5C). Given that the anti-BrdU antibody only recognizes BrdU in ssDNA and not in dsDNA, labeling without DNA denaturation allows visualization of ssDNA fibers in cells (62). Figure 5D and Supplementary Figure S6 clearly showed an increase in BrdU staining in the nucleus of Ku-depleted cells 2h after DSBs production, suggesting an enhanced ssDNA production at DSBs in the absence of Ku.

#### DISCUSSION

By controlling the level of expression of C-NHEJ components, we have shown here that Ku counteracts mobilization of PARP1 and the MRN complex to chromatin damaged with DSBs in cells and limits PAR synthesis as well as ssDNA production in response to these lesions.

The PARP1 mobilization to damaged chromatin under Ku-deficiency that we report most likely relies on a direct recruitment to DSBs since we found that it is accompanied by PAR synthesis. PARP1 exhibits a high binding affinity for and is activated by double-stranded DNA ends *in vitro* (71,72) and in cells (73). In addition, biochemical experiments with purified proteins provide evidence for a competition between Ku complex and PARP1 for binding to DNA ends (34). However, no experiments have previously demonstrated biochemically competition between both DNA-ends binding activities in the presence of chromosomal DSBs.

The competition between Ku and PARP1 mostly relies on Ku as a DSB-binding complex per se and not on a secondary repair function of Ku. This is shown by the recovery of PARP1 in damaged chromatin, which was stronger in the absence of Ku than in the absence of DNA-PKcs or LIG4. As a result, it has been shown in cells that the contribution of PARP1 to repair of chromosomal DSBs was very faint in  $LIG4^{-/-}$  cells and undetectable in DNA-PKcs deficient cells, both conditions still allowing Ku binding to DSBs (34). In contrast, a PARP inhibitor strongly impacted DSB repair in Ku-defective cells (34,52). A similar model of competition for access to the double-stranded DNA ends has been proposed earlier between Ku and Rad52 for initiation of homologous recombination (74). However, we cannot exclude that Ku sequesters PARP1 from performing its function at DSBs.

Although calicheamicin generated  $\sim 70\%$  non-DSB lesions (66), we observed a much stronger mobilization of PARP1 to damaged chromatin in the absence of Ku than expected on  $\sim 30\%$  DNA damage (DSBs) that were freed as additional binding sites upon Ku removal. This could be explained by a higher affinity of PARP1 for DSB than for SSB (71,72). Alternatively, a slower turnover of PARP1 on DSB could explain this observation; this is suggested by a bias towards *trans*-poly(ADP-ribosylation) mediated by DSB in vitro, as opposed to auto-poly(ADPribosylation) necessary for PARP1 detachment from DNA (71). In addition to the Ku-mediated impairment of PARP1 recruitment at DSBs that we show here, it has been suggested that the frequent associated SSBs after treatment with DNA-breaking agents could also divert PARP1 from DSBs (34). This effect may be more pronounced for DSB inducers like ionizing radiation which generates a ~20-fold ratio of SSBs to DSBs, much higher than the  $\sim$ 3-fold ratio measured for

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**Figure 5.** Consequences of Ku deficiency on PAR synthesis and ssDNA production in damaged chromatin. (A) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated or treated with 40  $\mu$ M DPQ for 1 h at 37°C, then 1 mM H<sub>2</sub>O<sub>2</sub> was added to the medium for 15 min Cells were then fixed, permeabilized and immunostained with anti-PAR antibodies followed by incubation with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with DAPI. (B) MRC5 shKu70 incubated or not with doxycyclin for 8 days were mock-treated or treated with 40  $\mu$ M DPQ for 1 h at 37°C, then 40 nM Cali was added to the medium for 7 min Cells were fixed, permeabilized and immunostained with anti-PAR antibodies followed by incubation with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse IgG. DNA was stained with DAPI. (C and D) After incorporation of 20  $\mu$ M BrdU, MRC5 shKu70 incubated or not with doxycyclin for 8 days, mock-treated or treated with 10 nM Cali for 45 min and post-incubated for 2 h were subjected to immunofluorescence with anti-BrdU antibodies and Alexa-Fluor<sup>®</sup> 594-conjugated goat anti-mouse antibodies. In C, a DNA denaturation step was included by incubation in 2N HCl for 10 min prior to immunostaining with anti-BrdU antibodies. DNA was stained with DAPI.

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Figure 5. Continued.

calicheamicin (66). The recruitment of PARP1 to chromatin containing DSBs that was observed in the absence of Ku paralleled to some extent that of XRCC1/LIG3 proteins. In addition, LIG3 loading on damaged chromatin relied on PARP1 activity since it was prevented by a PARP inhibitor. Indeed, recruitment of the XRCC1/LIG3 complex onto damaged chromosomal DNA has been reported to require PARP activity (67,75).

We report here that Ku counteracts access of the nuclease MRE11 and its partners NBS1 and Rad50 to chromatin damaged with DSBs. In Saccharomyces cerevisiae, it has been suggested that Ku competes with exonucleases at DNA ends (76-78) and in Schizosaccharomyces pombe, the Ku heterodimer inhibits nucleolytic processing of DSB ends (79). In mammalian cells, the absence of Ku has been shown to increase nucleolytic degradation of transfected DNA (39,80) or intra-chromosomal substrate (42), although a role for MRE11 nuclease has not precisely been assessed in these studies. PARP1 exclusion by Ku most likely impacts negatively MRE11 and NBS1 loading to damaged chromatin: similarly to LIG3, we found that MRE11 recruitment was reduced by PARP1 inhibition. Accordingly, laser-induced mobilization of MRE11 and NBS1 to sites of DNA damage relies on PARP1 (81).

When recruited to damaged chromatin under Ku knock-down conditions, PARP1 partially inhibits the

remaining DNA-PK activity, as shown by stimulation of RPAp34 and XRCC4 phosphorylation by treatment with a PARP inhibitor. Conversely, we found a slight contribution of DNA-PKcs activity to the impairment of PARP1 recruitment to DSBs. Thus, this supports a model in which the balance between DNA-PK and PARP1 at DSB sites is regulated both by a Ku/PARP1 competition for access to the DNA-ends, and also by a negative effect of each of the protein kinase or ADP-ribosylase activities on the competing one: under normal conditions, PARP1 binding to, and activation by DSBs may be competed by Ku and further downregulated by DNA-PK; conversely, DNA-PK activity may be competed by PARP1 under limiting conditions for Ku access to DSBs.

We found that Ku reduction in cells induces a shift from C- to B-NHEJ as revealed in a plasmid end-joining assay with cells extracts and that addition of purified Ku to extracts from Ku-depleted cells reversed this shift. Also, we have reported that LIG4 defective extracts do not show plasmid end-joining unless Ku but not DNA-PKcs is depleted from the extracts and that then, end-joining relied on B-NHEJ (26). In agreement, specific loss of Ku, among other C-NHEJ factors, has been reported to allow the best rescue by alternative mechanisms of the DSB repair defect. In plasmid-rejoining assays in human HCT116 human cells, inactivation of DNA-PKcs, Cernunnos-XLF or LIG4 yielded only 1-10% of the repair events measured after Ku80 genetic ablation, which reached levels even slightly higher than in wild-type cells (39). In the same study, it was shown that the strong repair defect in C-NHEJ mutants other than Ku could be rescued by reducing the amount of Ku in these cells (39). Similarly, joining efficiency of an intra-chromosomal substrate in hamster cells was strongly reduced in XRCC4-defective cells but close to normal in Ku-deficient cells (37); in MEF cells, plasmid end-joining in a transfection assay was compromised by XRCC4 defect but not by lack of Ku (36). Also, deletion of Ku70 was shown to rescue the sensibility to ionizing radiation of LIG4-defective DT40 cells (82) and Ku knock-out restored the viability of LIG4-null mice (83). Together with findings presented here, these results emphasize the predominant role of Ku as compared to other C-NHEJ proteins for counteracting access to DSBs of non C-NHEJ activities.

In addition to the two well characterized mammalian pathways for DSB repair, namely HR and C-NHEJ, recent reports have revealed a robust alternative endjoining or back-up mechanism (B-NHEJ) in C-NHEJ-deficient cells, operating efficiently on linear plasmid (84, 85),intra-chromosomal substrates (37,38,42,44,51), CSR-associated DSB (27,30,40,86) or radiation-induced DSBs (33,87). Any alternative DSB end-joining mechanism would be expected to require at least DNA end-binding and bridging, ends-processing and final ligation steps. Indeed, studies including our own have reported that in the absence of Ku, PARP1 can achieve synapsis (52) and PARP1/XRCC1-LIG3 can promote end-joining (34,44,52,53,55,56). In addition, several recent studies have implicated the MRN complex in B-NHEJ in mammalian cells, *via* end-tethering and/or nuclease activities, possibly associated with DSB signaling functions (88). We report here an excess of ssDNA production in the presence of DSBs under conditions of Ku deficiency but other experiments are needed to attribute this production to resection or unwinding at DNA ends. Nevertheless, given that Ku removal allows the best conditions for alternative end-joining, the repair proteins preferentially associated with damaged chromatin under these conditions are good candidates for components of these alternative repair routes. Thus our results strongly support the involvement of PARP1, the MRN complex and possibly XRCC1-LIG3 in B-NHEJ. In addition, they favor a model of PARP1 as a scaffold for the recruitment of both resection and ligation activities in alternative end-joining of DSBs.

In conclusion, our results support a predominant role for Ku among other C-NHEJ proteins as a competing factor in cells against other DNA end-binding activities and the involvement at least of PARP1 and MRN in non C-NHEJ alternative end-joining routes. In the near future, our cellular model for conditional induction of a shRNA against Ku70 should allow us to characterize other cellular factors recruited at sites of DSB in the absence of Ku and to further establish players in the B-NHEJ pathway for the repair of DNA DSBs.

#### ADDENDUM

The sequence of the shRNA targeting Ku70 mRNA mentioned in the first paragraph of 'Expression vectors, cell transfection and transduction' in the 'Materials and Methods' section is from the reference: Biard,D.S. (2007) Untangling the relationships between DNA repair pathways by silencing more than 20 DNA repair genes in human stable clones. *Nucleic Acids Res.*, **35**, 3535–3550.

#### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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