Published in final edited form as:

DNA Repair (Amst). 2012 March 1; 11(3): 310–316. doi:10.1016/j.dnarep.2011.12.007.

Persistently bound Ku at DNA ends attenuates DNA end resection and homologous recombination

Zhengping Shao^{1,2,3}, Anthony J. Davis^{1,3}, Kazi R. Fattah^{1,3}, Sairei So¹, Jingxin Sun¹, Kyung-Jong Lee¹, Lynn Harrison⁴, Jun Yang^{2,5}, and David J. Chen^{1,5}

¹Division of Molecular Radiation Biology, Department of Radiation Oncology, The University of Texas Southwestern Medical Center, 2201 Inwood Rd, Dallas, Texas, 75390

²The First Affiliated Hospital, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, Hangzhou, Zhejiang 31003, China

⁴Department of Molecular and Cellular Physiology, LSUHSC-S, 1501 Kings Highway, Shreveport, LA 71130

Abstract

DNA double strand breaks (DSBs) are repaired by non-homologous end joining (NHEJ) or homologous recombination (HR). The DNA cell cycle stage and resection of the DSB ends are two key mechanisms which are believed to push DSB repair to the HR pathway. Here, we show that the NHEJ factor Ku80 associates with DSBs in S phase, when HR is thought to be the preferred repair pathway, and its dynamics/kinetics at DSBs is similar to those observed for Ku80 in non-S phase in mammalian cells. A Ku homolog from *Mycobacterium tuberculosis* binds to and is retained at DSBs in S phase and was used as a tool to determine if blocking DNA ends affects end resection and HR in mammalian cells. A decrease in DNA end resection, as marked by IR-induced RPA, BrdU, and Rad51 focus formation, and HR are observed when Ku deficient rodent cells are complemented with Mt-Ku. Together, this data suggests that Ku70/80 binds to DSBs in all cell cycle stages and is likely actively displaced from DSB ends to free the DNA ends for DNA end resection and thus HR to occur.

Keywords

Homologous Recombination; Non-homologous end-joining; Ku70/80; DNA end resection; DNA double-strand breaks

1. INTRODUCTION

DNA double strand breaks (DSBs) are caused by endogenous (byproducts of cellular metabolism and replication associated errors) and exogenous (ionizing radiation and

© 2012 Elsevier B.V. All rights reserved.

⁵Corresponding author: Professor and Director, Division of Molecular Radiation Biology, Department of Radiation Oncology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9187, Phone (214)-648-5597, FAX (214)-648-5995, david.chen@utsouthwestern.edu.

³These authors contributed equally to the study

Conflict of Interest: The authors report no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

chemotherapeutic drugs) agents [1]. Unrepaired or misrepaired DSBs can result in senescence, induced apoptosis, or chromosomal aberrations and ultimately to genomic instability and tumorigenesis. To counteract the effects of DSBs, two highly efficient DSB repair pathways have evolved in eukaryotic cells: non-homologous end-joining (NHEJ) and homologous recombination (HR)[2]. NHEJ is initiated by the association of the Ku70/80 heterodimer to DNA ends. The Ku heterodimer is a protein with a ring-shaped structure that displays an extraordinary affinity for open DNA ends and is the initial sensor of the DSB [3]. The DNA–Ku70/80 complex then functions as a scaffold to assemble the other key NHEJ proteins at the DNA termini [4–6]. After end processing, the two tethered DNA termini are ligated and NHEJ is complete. HR is initiated once 5'-3' resection of the DSB occurs [7]. The initial sensor and processor of the DSB for the HR pathway is likely the Mre11-Rad50-Xrs2/Nbs1 (MRX/N) complex as this complex binds to DSBs and the Mre11 subunit has both endonucleases and exonuclease activity *in vitro* [8]. A number of other factors have been implicated in being required for DNA end resection, including CtIP (Sae2 in yeast), Exo1, and the Bloom protein (Sgs1 in yeast)[9–10]. Resection generates recombinogenic 3' single-strand tails which are stabilized by replication protein A (RPA). RPA is subsequently replaced by Rad51 to allow the initiation of pairing and strand invasion with homologous duplex DNA and HR is completed.

One of the major unresolved questions in the field of DNA repair is the mechanism that modulates the pathway choice between NHEJ and HR for the repair of DSBs. Two factors are believed to play major roles in the choice of HR over NHEJ [11]. The cell cycle phase of the cell is the first important factor in determining DSB repair pathway choice as HR requires a homologous DNA template; therefore, HR is thought to only be active during S and G2 phases of the cell cycle when a sister chromatid is available. NHEJ results in the direct ligation of the two ends of the broken DNA molecule. This process does not have the need for a homologous template and is therefore theoretically not restricted to a certain phase of the cell cycle, but it is assumed to be the dominant repair pathway in G0 and G1 phases. The second factor which likely modulates the pathway choice between HR and NHEJ is DNA end resection and direct competition between the pathways for the DSB and its repair. The prevalent model for DNA end resection and direct competition between the two pathways comes from genetic and biochemical data generated from studies with *Saccharomyces cerevisiae*. NHEJ (Ku70/80) and HR (MRX/N) factors directly compete for the same DNA double strand end *in vivo* and in S/G2 phase the DNA end resection machinery quickly forms ssDNA ends which initiates HR [9–10, 12]. The competition model is further supported by the fact that Ku and the MRX/N complex bind independently and almost simultaneously to DSBs and the lack of either complex results in an increase in the binding of the other complex and thus an increase in the other repair pathway [13–15]. Recent data in yeast further back a direct competition model between NHEJ and HR for DSBs [16–18]. Biochemical data suggests that the MRX complex can block the recruitment of Ku to DSBs and if Ku is bound to DSBs, the MRX complex can actively displace Ku for DNA end resection to occur. Although the use of *S. cerevisiae* as a model organism is powerful, it is likely that DSB-mediated repair and pathway choice in yeast and mammalian cells are not equivalent. First, the MRX complex is required for classical NHEJ in yeast, but it appears it is only required for an alternative end-joining pathway in mammalian cells. Second, DNA-PKcs is not found in yeast cells and DNA-PKcs likely plays an important role with Ku70/80 in protecting DNA ends from resection factors and mediating pathway choice.

In this report, we wanted to test if Ku localized to DSBs and determine its dynamics/kinetics at DSBs in S phase of the cell cycle in mammalian cells. We found that Ku70/80 localizes to DSBs in S phase and that the dynamics of Ku80 at DSBs are similar in S and non-S phase cells which suggests that Ku may play a general, initial role at DSBs in all cell cycle phases. Furthermore, we found that a homolog of Ku70/80 from *Mycobacterium tuberculosis* (Mt-

Ku) localizes to DSBs and is persistently retained at the DSB site in S phase. As Ku80 was able to bind to DSBs in S phase, it is likely that a mechanism mediates its dissociation from DSB in S phase for HR-mediated repair to occur. As the Mt-Ku protein was found to be persistently bound to DSB in S phase, this protein was used as a biological tool to determine if persistent retention of a Ku-like protein interferes with DNA end resection and HR-mediated repair and gain insight into the requirement of free ends for the HR pathway. Complementing Ku deficient rodent cell lines with Mt-Ku results in a marked decrease in the recruitment of the HR factors, RPA and Rad51, to IR-induced DSBs and ultimately, HR-mediated DSB repair. This data provides evidence that Ku70/80 localizes to DSBs in all phases of the cell cycle and must be actively displaced from DSB ends to allow DNA end resection and thus HR-mediated repair to occur.

2. MATERIALS AND METHODS

2.1. Cell culture and transfections

The Chinese hamster ovary Ku80 deficient cell line Xrs5 and the Ku70/80 double knock-out (DKO) mouse embryo fibroblasts (MEF) were cultured in Hyclone MEM media containing 10% Fetal Bovine Serum and Newborn Calf Serum (1:1 mixture), 100U/ml penicillin and 100U/ml streptomycin. The cells were incubated 37°C in a humidified incubator with 5% CO₂. Xrs5 cells stably integrated with the DR-GFP vector were grown in the medium described above plus 200µg/ml hygromycin. Cell lines containing both the DR-GFP vector and Flag-Ku constructs were grown in 200µg/ml hygromycin + 500µg/ml G418. All transfections were performed using the Nucleofector® II Kit (AmaxaBiosystems) or Eugene6 (Roche) according to the manufacturer's instructions. Ku70/80 DKO MEFs were transfected using Amexa program X-001 with 4µg of either YFP-tagged Ku80 or GFP-tagged Mt-Ku with 2µg of Ds-Red PCNA. Xrs5 cells were transfected using Eugene 6 with 1µg of either YFP-tagged Ku80 or GFP-tagged Mt-Ku with 1 µg of Ds-Red PCNA. Xrs-5 cells were also transfected using Amaxa Biosystems program U-027.

2.2. Live cell imaging and laser micro-irradiation

Live cell imaging combined with laser micro-irradiation was carried out as described previously with modifications [4, 19]. Fluorescence was monitored by using an Axiovert 200M microscope (Carl Zeiss, Inc), with a Plan-Apochromat 63X/NA 1.40 oil immersion objective (Carl Zeiss, Inc). A 365-nm pulsed nitrogen laser (Spectra Physics) was directly coupled to the epifluorescence path of the microscope and used to generate DSBs in a defined area of the nucleus. For quantitative analyses, standardized micro-irradiation conditions (minimal laser output of 75% for 5 pulses) were used to generate the same amount of DNA damage in each experiment. Time-lapse images were taken by an AxioCamHRm camera and the fluorescence intensities of micro-irradiated and non-irradiated areas within the cell nucleus were determined using the Axiovision Software, version 4.8 (Carl Zeiss, Inc). To eliminate the influence of nuclear background fluorescence, the fluorescence intensity of an undamaged site in the same nuclei was subtracted from the fluorescence intensity of the accumulation spot for every cell at each time point. Nonspecific photobleaching and UV lamp output fluctuation were compensated for by correcting the accumulation site fluorescence intensity (IN) of each time point based on pre-laser background intensity using the formula: $IN(t) = Id_t / Ib_t * Ib_{preIR}$, where Id_t represents the difference between the accumulation spot intensity and the undamaged site background intensity of each time point, Ib_t represents the background intensity of each time point, and Ib_{preIR} represents the background intensity before irradiation. Relative fluorescence intensity (RF) was calculated using the formula: $RF(t) = (IN_t - IN_{preIR}) / (IN_{max} - IN_{preIR})$, where IN_{preIR} means IN of the micro-irradiated area before laser damage and IN_{max} is the

maximum IN in the micro-irradiated area of all time points. Each data point is the average of 10 independent measurements.

2.3. Ku from *Mycobacterium tuberculosis*

Two open reading frame expression vectors encoding the Ku homolog from *Mycobacterium tuberculosis* (Mt-Ku) were used (as outlined in [20]). Mt-Ku does not localize to the nucleus in eukaryotic cells so the nuclear localization signal (NLS) from human Ku70 (amino acids 539–556 KVTRKHDNEGSGSKRPK) was added to target the Mt-Ku protein to the nucleus. Additionally, an EGFP (KuEnls) or FLAG tag was added downstream of the Mt-Ku coding sequence. In this manuscript, the Mt-Ku-NLS-EGFP is referred to as GFP-Mt-Ku and the Mt-Ku-NLS-FLAG as FLAG-Mt-Ku.

2.4. Fluorescent immunostaining

Following irradiation with 8 Gy, foci formation of RPA and Rad51 were detected using fluorescent immunostaining at various time points as described in the figure legends. For immunostaining, cells were pre-extracted for 5 minutes on ice in PBS containing 0.1% TritonX-100 (or 1% Triton X-100 for Rad51 staining of Xrs5 cells), fixed in PBS with 4% formaldehyde for 15 minutes, permeabilized in 0.25% Triton X-100 for 5 minutes (1% Triton X-100 for Rad51 staining of Xrs5 cells), blocked with PBS containing 5% normal goat serum overnight at 4°C, and finally incubated with the primary antibodies, RPA2 mouse monoclonal antibody (Calbiochem, NA19L) and Rad51 rabbit polyclonal antibody (Santa Cruz, H1810). Signals were visualized by use of secondary antibodies conjugated with Alexa488 (Invitrogen, A11001) or Texas red (Invitrogen, T2767). To detect ssDNA formation by BrdU foci staining, cells were cultured in 30μM BrdU (Roche) for 36 hours before IR. Following irradiation with 20 Gy, foci formation of BrdU were detected using fluorescent immunostaining at various time points. The cells were pre-extracted in PBS containing 0.25% Triton X-100 on ice for 5 min, fixed in 70% methanol and 30% ethanol at –20°C for 30min, then permeabilized in 1% Triton X-100/PBS on ice for 10min, blocked with NGS buffer (5% normal goat serum, 0.5% Tween 20, and 0.1% BSA in PBS) at 37°C for 40min, and probed with mouse monoclonal anti-BrdU primary antibody (BD Biosciences) and secondary antibodies conjugated with Texas red (Invitrogen, T2767) at 37°C.

2.5. HR assay

Cell lines were transfected with 5ug RFP-ISceI-GR construct to induce I-SceI expression using the Amaxa protocol described in the Sup. Methods. Cells were cultured in complete medium with 0.1μM Triamcinolone acetonide (TA) to induce translocation of the RFP-ISceI-GR construct to the nucleus. Cells were analyzed by flow cytometry 48 hours after transfection. %HR was calculated as $\text{GFP}^+ / (\text{RFP}^+ + \text{GFP}^+)$. Xrs5-DR6 cells were also transfected with GFP and RFP plasmids to be used as controls.

3. RESULTS AND DISCUSSION

3.1. Human Ku80 and Mt-Ku localize to DSBs in S phase

In mammalian cells, NHEJ is believed to be the dominant DSB repair pathway in G0 and G1 phases of the cell cycle whereas HR is the dominant pathway in S and G2 phases. To expand upon a possible role for Ku70/80 at DSBs in S phase, a micro-irradiation system was used to verify if Ku70/80 localized to DSBs and determine its dynamics/kinetics at DSBs in S phase in mammalian cells [21–22]. YFP-tagged Ku80 was transiently expressed in the Ku80-deficient Chinese hamster ovary (CHO) cell line Xrs5 along with Ds-Red tagged PCNA. Complementation of Ku80 deficient CHO cells with human Ku80 corrects the

radiosensitivity of the Ku80 deficient cell line and interacts with the hamster Ku70 and restore hamster Ku70's ability to interact with DNA, thus localization of YFP-tagged human Ku80 to laser-generated DSBs is a sensitive method to measure the ability of the Ku70/80 heterodimer to localize to DNA damage [23]. Ds-Red tagged PCNA was expressed to allow for the differentiation of S and non-S phase cells as PCNA shows a faint and even distribution in non-S phase cells whereas it forms distinct foci in S phase (Fig. 1A and B and Sup. Fig. 1)[24]. To study the dynamics of YFP-tagged Ku80 at DSBs in living cells, a live cell imaging system coupled to a 365-nm laser micro-irradiation system was utilized [4, 19]. Using this system, we observe that Ku80 rapidly localizes to DSBs in S phase (Fig. 1A) and non-S phase cells (Sup. Fig. 1A and data not shown). Time-lapse imaging showed that the kinetics of Ku80 in S and non-S phase cells are similar with initial recruitment and fluorescence intensity peaking at 10 minutes and 80% of the protein dissociated after 60 minutes and 95% after 120 minutes (Figure 1C and data not shown). The data shows that Ku70/80 binds to DSB ends and its kinetics/dynamics at DSBs are the same regardless of the cell cycle stage in mammalian cells. We hypothesize that Ku70/80 likely plays a universal role at DSBs which is independent of NHEJ, in that it binds to DSBs in all phases of the cell cycle to help maintain their stability by protecting them from non-specific processing and depending on a number of factors either mediates NHEJ or is actively displaced for DNA end resection and HR to occur. The universal role of Ku70/80 in stabilizing DSB ends regardless of the cell cycle stage may explain its strong affinity for DNA ends ($2.4 \times 10^9 \text{ M}^{-1}$) and its ability to bind to a number of different DSB species including those with blunt ends, 3' and 5' overhangs, and ends with hairpins [25–26]. Lastly, a role for Ku70/80, but not the MRN complex, in stabilizing DNA ends is supported by evidence which shows that Ku80 is required for the positional stability of single DSBs in asynchronous mammalian cells [27].

We have shown that a homolog of Ku70/80 from *Mycobacterium tuberculosis* (Mt-Ku) localizes to laser-generated DSBs in both human and hamster cells [20]. Mt-Ku is a 30-kDa protein, which forms a homodimer and is similar to Ku70/80 as it binds to dsDNA ends and mediates end-joining of DSBs [28–30]. To further expand on this, using the system described above, the recruitment and real-time kinetics of GFP-tagged Mt-Ku to laser-induced DSBs in Xrs5 and Ku70/80 double knock-out MEFs (Ku DKO) in S and non-S phase of the cell cycle was monitored. Ku DKO MEFs were used because it eliminates any possible endogenous Ku expression. Mt-Ku localizes to laser-generated DSBs in S and non-S phase cells (Fig. 1B) and real-time kinetics show that Mt-Ku reaches its peaks localization between 20–30 minutes post micro-irradiation in Xrs5 (Fig. 1C) and Ku DKO (Fig. 1D) cells. Surprisingly, unlike human Ku80, Mt-Ku does not rapidly dissociate from the laser-induced DSBs in S or non-S phase cells in either cell line. After 60 minutes, roughly 80% of Ku80 had dissociated from the DSBs, whereas only 20% of the Mt-Ku had dissociated from the DSBs. We hypothesize that Mt-Ku is retained at DSBs in non-S phase cells because it is not able to interact with DNA-PKcs and thus cannot mediate NHEJ-mediated repair of DSBs [20]. In S phase, we hypothesize that the mechanism which mediates Ku's dissociation from S phase cell fails to recognize Mt-Ku.

3.2. Blocking of DNA ends attenuates DNA end resection and recruitment of HR proteins to DSBs

One of the key requirements for determining between the DSB repair pathways NHEJ and HR in eukaryotic cells is whether DNA ends are free to allow 5'–3' resection. Previous work in *S. cerevisiae* has shown that deletion of yKu results in an increase in resection of endonucleolytic created DSBs in G1, suggesting that DNA ends pre-bound by Ku blocks DNA end resection [18, 31–32]. Ku80 localizes to DSBs in a similar fashion in S and non-S phase cells; therefore, we hypothesize that Ku must be actively displaced from DSB in S/G2

phases of the cell cycle for DNA end resection to occur. As Mt-Ku was retained at laser-generated DSBs in S phase of the cell cycle, it was tested if retention of this protein at DSBs results in a defect in DNA end resection in mammalian cells. DNA end resection results in the generation of ssDNA ends which are rapidly coated by RPA and formation of DNA damaged-induced RPA foci is an established indirect method for observing the formation of ssDNA and thus DNA end resection [33]. To test if DNA end resection was attenuated when DSB ends were blocked by an immobile Ku-like protein, Ds-Red tagged Mt-Ku was expressed in Ku DKO cells, irradiated with 8 Gy, and immunofluorescent assays were performed to determine RPA foci formation in the cells compared to uncomplemented Ku DKO cells and wild-type (WT) MEFs. The percentage of cells containing >5 RPA foci is markedly reduced in the Ku DKO cells expressing Mt-Ku compared to the Ku DKO and wild-type MEF cells 1, 4, and 8 hours following DNA damage (Fig. 2A). Furthermore, the average RPA foci number/cell is reduced in the Ku DKO cells expressing Mt-Ku (Sup. Fig. 2). RPA focus formation in Xrs5 cells complemented with Mt-Ku was not performed because RPA antibodies do not recognize hamster RPA (data not shown). In addition, generation of ssDNA at the sites of DSBs can be detected directly by immunostaining with BrdU without previous DNA denaturation [34]. Using this technique, it was observed that following treatment with 20 Gy of IR, the percentage of cells with >5 BrdU foci was less in the Ku DKO cells expressing Mt-Ku compared to the Ku DKO and WT cells 1, 2, and 4 hours post IR treatment (Fig. 2B). Finally, Rad51 replaces RPA on the ssDNA and is required for strand invasion for HR to occur. Expression of Mt-Ku in the Ku DKO cells results in a marked decrease in the number of cells containing Rad51 foci 1, 4, and 8 hours following treatment with 8 Gy of IR compared to the Ku deficient cells and the wild-type mouse cells (Fig. 3A). Similarly, expression of Mt-Ku in Xrs5 cells results in a decrease in IR-induced Rad51 foci (Fig. 3B). Taken together, this data suggests that DNA end resection and subsequently recruitment of HR factors to DSBs is attenuated when DNA ends are blocked by Mt-Ku. Consistent with this, pre-binding of a model DNA substrate with Mt-Ku blocks Exo1-mediated DNA end resection (Sup. Fig. 3), suggesting that Mt-Ku can block DNA end resection *in vivo* and *in vitro*. The data suggests that in mammalian cells DSB ends must be free for resection to push DSB repair pathway choice to HR-mediated repair.

3.3. DSB ends must be free for HR-mediated repair

The data presented above suggests that expression of Mt-Ku affects DNA end resection, which should then lead to a reduction in HR-mediated DSB repair. To confirm that cells expressing Mt-Ku results in a decrease in HR, we utilized an *in vivo* HR assay [35]. This established system uses a GFP recombination reporter substrate with an I-SceI restriction site which when a DSB is produced via the I-SceI restriction endonuclease produces GFP fluorescence only if HR-mediated repair occurs using a fragment of GFP in the reporter substrate as a homologous sequence (Figure 4A). Cell lines were isolated in which only one copy of the HR reporter assay vector (DR-GFP) was integrated into the Xrs5 genome (Xrs5-DR6) and then stably complemented with either FLAG-tagged Ku80 or Mt-Ku (Sup. Fig. 4). Finally, a chimera between the I-SceI restriction endonuclease and the ligand-binding domain of the glucocorticoid receptor in frame with monomeric RFP was used (RFP-I-SceI-GR) [27]. The advantage of using this RFP-I-SceI-GR chimera is that the RFP can be used to determine transfection efficiency and is cytoplasmic in the absence of the glucocorticoid triamcinolone acetonide (TA) and translocates rapidly to the nucleus when TA is added. To perform the assay, each cell line was transiently transfected with the RFP-I-SceI-GR construct and TA was added. Cells were analyzed by flow cytometry and % HR was calculated. Similar to previous results, HR-mediated repair is increased in the absence of Ku (32.05%) compared to cells expressing human Ku (6.45%) (Fig. 4B)[35]. The data clearly shows that when DSBs are persistently bound by Mt-Ku, a marked decrease (14.45%) in HR-mediated DSB repair is observed when compared to the Ku deficient cells (32.05%).

HR was higher in the cells complemented with Mt-Ku than human Ku80 which likely occurs because Mt-Ku does not interact with DNA-PKcs and thus cannot mediate NHEJ-mediated repair of the I-SceI induced DSB [20]. As complementation of CHO deficient cells with human Ku can mediate NHEJ, a percentage of the I-SceI induced DSBs are repaired via NHEJ and thus are not observed in our assay as being repaired and results in a smaller % of cells able to be repaired by HR [23]. Together, the data shows that DNA ends must be free for DNA end processing and HR-mediated repair to occur cells.

3.4. CONCLUSIONS AND MODEL

Our data shows that in mammalian cells, the NHEJ factor Ku80 associates with DSBs in S phase, the cell cycle phase when HR is thought to be the preferred repair pathway, and that its initial localization and dynamics at DSBs is similar to those observed with Ku80 in non-S phase cells. A Ku homolog from *Mycobacterium tuberculosis* was used as a tool to determine the biological consequences of persistently blocking DNA ends by a Ku70/80-like protein. Complementation of Ku deficient rodent cells with Mt-Ku results in attenuated DNA end resection and HR-mediated repair, suggesting that DNA ends must be free for HR-mediated DSB repair. Taken together, we suggest the following model for DSB repair and pathway choice in S phase in mammalian cells. Following the creation of a DSB, Ku70/80 binds to the DNA, possibly in conjunction with DNA-PKcs, to protect the end from non-specific processing until it is actively displaced from the DSB for HR to occur. The protecting of DNA ends by Ku70/80 occurs in all cell cycle phases and is independent of its role in NHEJ-mediated repair. If Ku70/80 is not displaced, DNA end resection will be attenuated and HR will not occur. This is supported by data in yeast that shows that the DNA end resection process is hindered if the Ku protein is not first properly removed from DSB ends [10, 13]. This is further supported by data presented in this paper which shows that the active blocking of DNA ends results in attenuation of DNA end resection and formation of ssDNA overhangs as a marked decrease in the number of RPA (Figure 2A) and BrdU (Figure 2B) foci are observed when Ku deficient cells are complemented with Mt-Ku which is persistently bound to DSBs in S phase. We speculate that Ku70/80 is actively displaced from DSBs in S/G2 phases of the cell cycle for HR to occur. A number of mechanisms may be able to actively displace Ku from DSB ends. In yeast, it is believed that MRX may play a direct role in removing Ku from DNA ends. *In vitro* studies have shown that Ku pre-bound to DNA can block Exo1-mediated DNA end resection and that addition of MRX/Sae2 partially recovers Exo1-mediated resection suggesting the MRX complex mediates the dissociation of Ku from DNA ends [18]. Furthermore, post-translational modifications may play a role in mediating the active dissociation of Ku from DNA ends. DSB-induced ubiquitylation of Ku80 results in its dissociation from DNA *in vitro* and poly adenosine diphosphate (ADP)-ribosylation (PARylation) mediates retention of Ku to DSBs [36–37]. Although these mechanisms are interesting, it is still not known if they mediate the dissociation of human Ku from DSBs and if this dissociation is required for HR-mediated repair in S/G2 phases of the cell cycle. It is likely that the mechanism which mediates the displacement of Ku from DSBs is not found in the Mt-Ku protein and the retention of the Mt-Ku to laser-generated DSBs is due to this. Future work will need to be performed to determine the mechanism mediating the dissociation of Ku from DSBs in late S/G2 phases of the cell cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful to Austin DeBeaux and Reneau Castore from the Harrison laboratory for the creation of the Mt-Ku-EGFP and NLS fusion and FLAG-Mt-Ku-NLS open reading frame expression vectors. The research was supported by National Institutes of Health grants (CA50519, CA134991, and CA92584) and Cancer Prevention Research Institute of Texas (RP110465) to DJC and National Institutes of Health grants are CA85693 and CA85693-9S1 to LH. ZS was supported by a fellowship from the China Scholarship Council.

ABBREVIATIONS

DSBs	DNA double strand breaks
NHEJ	non-homologous end joining
HR	homologous recombination
MRX/N	Mre11-Rad50-Xrs2/Nbs1
Mt-Ku	Ku homolog from <i>Mycobacterium tuberculosis</i>
CHO	Chinese hamster ovary
Ku DKO	Ku70/80 double knock-out mouse embryonic fibroblasts
RPA	replication protein A
PCNA	proliferating cell nuclear antigen
TA	triamcinolone acetonide

References

1. Khanna KK, Jackson SP. DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet.* 2001; 27:247–254. [PubMed: 11242102]
2. Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst).* 2006; 5:1042–1048. [PubMed: 16822724]
3. Walker JR, Corpina RA, Goldberg J. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature.* 2001; 412:607–614. [PubMed: 11493912]
4. Uematsu N, Weterings E, Yano K, Morotomi-Yano K, Jakob B, Taucher-Scholz G, Mari PO, van Gent DC, Chen BP, Chen DJ. Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. *J Cell Biol.* 2007; 177:219–229. [PubMed: 17438073]
5. Yano K, Morotomi-Yano K, Wang SY, Uematsu N, Lee KJ, Asaithamby A, Weterings E, Chen DJ. Ku recruits XLF to DNA double-strand breaks. *EMBO Rep.* 2008; 9:91–96. [PubMed: 18064046]
6. Mari PO, Florea BI, Persengiev SP, Verkaik NS, Bruggenwirth HT, Modesti M, Giglia-Mari G, Bezstarosti K, Demmers JA, Luiders TM, Houtsmuller AB, van Gent DC. Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proc Natl Acad Sci U S A.* 2006; 103:18597–18602. [PubMed: 17124166]
7. Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. *DNA Repair (Amst).* 2007; 6:923–935. [PubMed: 17363343]
8. Paull TT, Gellert M. The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell.* 1998; 1:969–979. [PubMed: 9651580]
9. Huertas P. DNA resection in eukaryotes: deciding how to fix the break. *Nat Struct Mol Biol.* 2010; 17:11–16. [PubMed: 20051983]
10. Niu H, Raynard S, Sung P. Multiplicity of DNA end resection machineries in chromosome break repair. *Genes Dev.* 2009; 23:1481–1486. [PubMed: 19571177]
11. Kass EM, Jasin M. Collaboration and competition between DNA double-strand break repair pathways. *FEBS Lett.* 2010; 584:3703–3708. [PubMed: 20691183]

12. Bernstein KA, Shor E, Sunjevaric I, Fumasoni M, Burgess RC, Foiani M, Branzei D, Rothstein R. Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair. *EMBO J.* 2009; 28:915–925. [PubMed: 19214189]
13. Wu D, Topper LM, Wilson TE. Recruitment and dissociation of nonhomologous end joining proteins at a DNA double-strand break in *Saccharomyces cerevisiae*. *Genetics.* 2008; 178:1237–1249. [PubMed: 18245831]
14. Delacote F, Han M, Stamato TD, Jasin M, Lopez BS. An *xrcc4* defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. *Nucleic Acids Res.* 2002; 30:3454–3463. [PubMed: 12140331]
15. Pierce AJ, Hu P, Han M, Ellis N, Jasin M. Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes Dev.* 2001; 15:3237–3242. [PubMed: 11751629]
16. Mimitou EP, Symington LS. Ku prevents Exo1 and Sgs1-dependent resection of DNA ends in the absence of a functional MRX complex or Sae2. *EMBO J.* 2010; 29:3358–3369. [PubMed: 20729809]
17. Paull TT. Making the best of the loose ends: Mre11/Rad50 complexes and Sae2 promote DNA double-strand break resection. *DNA Repair (Amst).* 2010; 9:1283–1291. [PubMed: 21050828]
18. Shim EY, Chung WH, Nicolette ML, Zhang Y, Davis M, Zhu Z, Paull TT, Ira G, Lee SE. *Saccharomyces cerevisiae* Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks. *EMBO J.* 2010; 29:3370–3380. [PubMed: 20834227]
19. So S, Davis AJ, Chen DJ. Autophosphorylation at serine 1981 stabilizes ATM at DNA damage sites. *J Cell Biol.* 2009; 187:977–990. [PubMed: 20026654]
20. Castore R, Hughes C, Debeaux A, Sun J, Zeng C, Wang SY, Tatchell K, Shi R, Lee KJ, Chen DJ, Harrison L. *Mycobacterium tuberculosis* Ku can bind to nuclear DNA damage and sensitize mammalian cells to bleomycin sulfate. *Mutagenesis.* 2011
21. Kim JS, Krasieva TB, Kurumizaka H, Chen DJ, Taylor AM, Yokomori K. Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. *J Cell Biol.* 2005; 170:341–347. [PubMed: 16061690]
22. Guirouilh-Barbat J, Huck S, Lopez BS. S-phase progression stimulates both the mutagenic KU-independent pathway and mutagenic processing of KU-dependent intermediates, for nonhomologous end joining. *Oncogene.* 2008; 27:1726–1736. [PubMed: 17891177]
23. Chen F, Peterson SR, Story MD, Chen DJ. Disruption of DNA-PK in Ku80 mutant *xrs-6* and the implications in DNA double-strand break repair. *Mutat Res.* 1996; 362:9–19. [PubMed: 8538653]
24. Leonhardt H, Rahn HP, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardoso MC. Dynamics of DNA replication factories in living cells. *J Cell Biol.* 2000; 149:271–280. [PubMed: 10769021]
25. Blier PR, Griffith AJ, Craft J, Hardin JA. Binding of Ku protein to DNA. Measurement of affinity for ends and demonstration of binding to nicks. *J Biol Chem.* 1993; 268:7594–7601. [PubMed: 8463290]
26. Mimori T, Hardin JA, Steitz JA. Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders. *J Biol Chem.* 1986; 261:2274–2278. [PubMed: 3511059]
27. Soutoglou E, Dorn JF, Sengupta K, Jasin M, Nussenzweig A, Ried T, Danuser G, Misteli T. Positional stability of single double-strand breaks in mammalian cells. *Nat Cell Biol.* 2007; 9:675–682. [PubMed: 17486118]
28. Della M, Palmbos PL, Tseng HM, Tonkin LM, Daley JM, Topper LM, Pitcher RS, Tomkinson AE, Wilson TE, Doherty AJ. *Mycobacterial* Ku and ligase proteins constitute a two-component NHEJ repair machine. *Science.* 2004; 306:683–685. [PubMed: 15499016]
29. Weller GR, Kysela B, Roy R, Tonkin LM, Scanlan E, Della M, Devine SK, Day JP, Wilkinson A, d'Adda di Fagagna F, Devine KM, Bowater RP, Jeggo PA, Jackson SP, Doherty AJ. Identification of a DNA nonhomologous end-joining complex in bacteria. *Science.* 2002; 297:1686–1689. [PubMed: 12215643]
30. Wright D, DeBeaux A, Shi R, Doherty AJ, Harrison L. Characterization of the roles of the catalytic domains of *Mycobacterium tuberculosis* ligase D in Ku-dependent error-prone DNA end joining. *Mutagenesis.* 2010; 25:473–481. [PubMed: 20530153]

31. Clerici M, Mantiero D, Guerini I, Lucchini G, Longhese MP. The Yku70–Yku80 complex contributes to regulate double-strand break processing and checkpoint activation during the cell cycle. *EMBO Rep.* 2008; 9:810–818. [PubMed: 18600234]
32. Zhang Y, Hefferin ML, Chen L, Shim EY, Tseng HM, Kwon Y, Sung P, Lee SE, Tomkinson AE. Role of Dnl4-Lif1 in nonhomologous end-joining repair complex assembly and suppression of homologous recombination. *Nat Struct Mol Biol.* 2007; 14:639–646. [PubMed: 17589524]
33. Raderschall E, Golub EI, Haaf T. Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proc Natl Acad Sci U S A.* 1999; 96:1921–1926. [PubMed: 10051570]
34. Meyn RE, Hewitt RR, Humphrey RM. Evaluation of S phase synchronization by analysis of DNA replication in 5-bromodeoxyuridine. *Exp Cell Res.* 1973; 82:137–142. [PubMed: 4270895]
35. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.* 1999; 13:2633–2638. [PubMed: 10541549]
36. Postow L, Ghenoiu C, Woo EM, Krutchinsky AN, Chait BT, Funabiki H. Ku80 removal from DNA through double strand break-induced ubiquitylation. *J Cell Biol.* 2008; 182:467–479. [PubMed: 18678709]
37. Couto CA, Wang HY, Green JC, Kiely R, Siddaway R, Borer C, Pears CJ, Lakin ND. PARP regulates nonhomologous end joining through retention of Ku at double-strand breaks. *J Cell Biol.* 2011; 194:367–375. [PubMed: 21807880]

HIGHLIGHTS

1. Ku localizes to and its kinetics at DSBs is similar in S and non-S phase cells.
2. A homolog of Ku from *Mycobacterium tuberculosis* is persistently retained at DSBs.
3. Blocking DNA ends with immobile Ku attenuates DNA end resection and HR.
4. DNA ends must be free for DNA end resection and HR-mediated DSB repair.

Figure 1. Ku80 and Mt-Ku localize to and Mt-Ku is retained at laser-generated DSBs in S phase (A) YFP-tagged Ku80 and (B) GFP-tagged Mt-Ku were transiently expressed in Xrs5 cells with DsRed-tagged PCNA. S phase and non-S phase cells were differentiated by the localization pattern of DsRed-PCNA. Localization of Ku80 or Mt-Ku and DsRed-PCNA to laser-generated DSBs are marked by a white arrow. **C.** Kinetics of relative fluorescent intensity of YFP-tagged human Ku80 and GFP-tagged Mt-Ku in Ku80 deficient CHO Xrs5 cells at DSBs after micro-irradiation in S phase cells. **D.** Kinetics of relative fluorescent intensity of GFP-tagged Mt-Ku in Ku70/80 double knock-out MEFs at DSBs after micro-irradiation in S and non-S phase cells.

Figure 2. DNA end resection is attenuated when DSB ends are blocked by Mt-Ku

A. RPA foci formation following DNA damage is attenuated in Ku70/80 double knock-out MEFs (Ku DKO) complemented with Mt-Ku. Immunostaining of wild-type MEFs, Ku DKO, or Ku DKO cells expressing Ds-Red Mt-Ku after 8 Gy of γ -ray. Cells were pre-extracted and fixed 1, 4, or 8 hours after irradiation and immunostained for RPA. RPA foci were counted per cell and the percent of cells with >5 RPA foci was quantitated. **B.** Ku DKO cells complemented with Mt-Ku results in attenuation of formation of ssDNA. Immunostaining of wild-type MEFs, Ku DKO cells, or Ku DKO cells expressing DsRed Mt-Ku after 20 Gy of γ -ray. Cells were pre-extracted and fixed 1, 2, or 4 hours after irradiation and immunostained with a specific antibody against BrdU. BrdU foci were counted per cell

and the percent of cells with >5 BrdU foci was quantitated. Error bars represent the standard deviation.

Figure 3. Recruitment of the HR factor Rad51 to DSBs is attenuated when DSB ends are blocked by Mt-Ku

Foci formation of Rad51 in (A) wild-type MEFs, Ku DKO cells, or Ku DKO cells expressing Ds-Red Mt-Ku and (B) Xrs5 cells and Xrs5 cells complemented with YFP-tagged Ku80 or GFP-tagged Mt-Ku after 8 Gy of γ -ray was observed. Cells were pre-extracted and fixed 1, 4, or 8 hours after irradiation and immunostained for Rad51. Rad51 foci were counted per cell and the percent of cells with >5 Rad51 foci was quantitated. Error bars represent the standard deviation.

Figure 4. Expression of Mt-Ku results in attenuation of HR-mediated repair

A. Cartoon of HR reporter assay. **B.** Xrs5 cells in which one copy of the HR reporter assay vector was integrated into the genome were either uncomplemented or complemented with FLAG-tagged human Ku80 or Mt-Ku. RFP-I-SceI-GR was transiently expressed and triamcinolone acetonide (TA) was added to mediate nuclearization of the RFP-I-SceI-GR and induce a DSB at the I-SceI restriction site. Cells were analyzed by flow cytometry and % HR was calculated as GFP/(RFP + GFP). Error bars represent standard deviation.