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

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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.

1. Introduction

DNA double strand breaks (DSBs) are caused by endogenous (byproducts of cellular metabolism and replication associated errors) and exogenous (ionizing radiation and chemotherapeutic drugs) agents [1]. Unrepaired or misrepaired DSBs can result in senescence, inducted 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

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 *Mycobacterium tuberculosis*; 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.

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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 *Sacchromyces 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 DBS 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), 100 U/ml penicillin and 100 U/ml streptomycin. The cells were incubated 37 °C in a humidified incubator with 5% CO_2. Xrs5 cells stably integrated with the DR-GFP vector were grown in the medium described above plus 200 μg/ml hygromycin. Cell lines containing both the DR-GFP vector and Flag-Ku constructs were grown in 200 μg/ml hygromycin + 500 μg/ml G418. All transfections were performed using the Nucleofector® II Kit (AmaxaBiosystems) or Fugene6 (Roche) according to the manufacturer's instructions. Ku70/80 DKO MEFs were transfected using Amaxa program X-001 with 4 μg of either YFP-tagged Ku80 or GFP-tagged Mt-Ku with 2 μg of Ds-Red PCNA. Xrs5 cells were transfected using Fugene 6 with 1 μg 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 Axiovison 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 M. tuberculosis

Two open reading frame expression vectors encoding the Ku homolog from *M. tuberculosis* (Mt-Ku) were used (at 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 KVTKRKHDNEGSGSKRPK) 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 min on ice in PBS containing 0.1% Triton X-100 (or 1% Triton X-100 for Rad51 staining of Xrs5 cells), fixed in PBS with 4% formaldehyde for 15 min, permeabilized in 0.25% Triton X-100 for 5 min (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) or Rad51 rabbit