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Heterozygous inactivation of human Ku70/Ku86 heterodimer does not affect cell growth, double-strand break repair, or genome integrity

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ABSTRACT

Ku, the heterodimer of Ku70 and Ku86, plays crucial roles in non-homologous end-joining (NHEJ), a major pathway for repairing DNA double-strand breaks (DSBs) in mammalian cells. It has recently been reported that heterozygous disruption of the human *KU86* locus results in haploinsufficient phenotypes, including retarded growth, increased radiosensitivity, elevated p53 levels and shortened telomeres. In this paper, however, we show that heterozygous inactivation of either the *KU70* or *KU86* gene does not cause any defects in cell proliferation or DSB repair in human somatic cells. Moreover, although these heterozygous cell lines express reduced levels of both Ku70 and Ku86, they appear to maintain overall genome integrity with no elevated p53 levels or telomere shortening. These results clearly indicate that Ku haploinsufficiency is not a commonly observed phenomenon in human cells. Our data also suggest that the impact of *KU70/KU86* mutations on telomere metabolism varies between cell types in humans.

1. Introduction

DNA double-strand breaks (DSBs) are one of the most severe lesions that if not repaired will lead to loss of genetic information resulting in cancer or cell death. DSBs can be caused by ionizing radiation (IR), reactive oxygen species, DNA replication across a nick, or the inhibition of DNA topoisomerase II [1]. Mammalian cells have evolved two major pathways for repairing DSBs, homologous recombination and non-homologous DNA end-joining (NHEJ) [2]. These pathways function cooperatively to maintain genomic stability in mammals [2]. NHEJ is the predominant pathway during G0, G1 and early S phases when no sister chromatid is available for homologous recombination [3].

Ku is a heterodimeric protein composed of 70 and 86 kDa subunits (Ku70 and Ku86, respectively), which can bind all sorts of double-stranded DNA ends in a sequence-non-specific fashion [4]. It has been established that Ku plays crucial roles in NHEJ [1,2,4]. When a DSB occurs, Ku recognizes and binds to DSB ends and subsequently recruits DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK complex, which is thought to phosphorylate and activate downstream targets, including Artemis [1,5]. After the trimming of DNA ends by the Artemis:DNA-PKcs complex and additional proteins, the XRCC4:DNA ligase IV complex is recruited to complete the joining [1,2].

Genetic studies using rodent or avian cells have shown that cells lacking Ku (and any of the core NHEJ components)

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display increased IR sensitivity [3,6–8], consistent with a role for NHEJ in DSB repair. Hypersensitivity of NHEJ-defective cells is more pronounced toward DSBs caused by topoisomerase II inhibitors, since such lesions are repaired primarily or exclusively by NHEJ [9,10]. Accumulating evidence also suggests that Ku deficiency can induce chromosome aberrations [11,12] and telomere dysfunctions [13–15]. It should be noted, however, that genetic deletion of either *Ku70* or *Ku86* does not result in lethality in rodent and avian cells, although growth retardation has been observed for Ku70- and Ku86-deficient animals [6–8,16,17].

Biochemical studies have demonstrated that human cells have higher levels of DNA-PK activity than do rodent cells, possibly suggesting a more vital role for Ku (and DNA-PKcs) in humans [18]. Indeed, no human patient with mutations in either Ku70 or Ku86 has been described, contrasting with the case for Artemis or DNA ligase IV [19,20]. Recently, Hendrickson and co-workers have shown that genetic deletion of the human *KU86* gene results in apoptotic cell death in the colon cancer cell line HCT116 [21], raising the possibility that Ku plays an essential role in human somatic cells. More intriguingly, the same group has reported that heterozygous disruption of the human *KU86* gene leads to growth retardation, IR hypersensitivity, and telomere shortening [21,22]. This finding suggests that even an ~50% reduction in cellular Ku86 levels might affect cell growth, DSB repair, and genome integrity in humans.

Given that Ku86 functions as Ku heterodimer, any phenotypes associated with *KU86* mutations could be attributed to the reduction or absence of Ku70/Ku86 heterodimer. However, it remains possible that a certain form of Ku86 protein has an essential cellular function independent of Ku70. A likely candidate for this is KARP-1, which is transcribed from the *KU86* locus with the use of a 5′ upstream promoter and additional exons and may regulate DSB repair [23]. Interestingly, KARP-1 expression appears to be restricted to primates [23]. Hence, for the purpose of clarifying the in vivo function of human Ku protein (i.e. the Ku70/Ku86 heterodimer), it is necessary to study the impact of mutations in *KU70*, in addition to (or rather than) *KU86*. In this paper, we generate by gene targeting *KU70*$^{+/-}$ cells as well as *KU86*$^{+/-}$ cells from the human pre-B acute lymphoblastic leukaemia cell line Nalm-6. We show that heterozygous inactivation of the *KU70* or *KU86* gene does not cause any defects in cell proliferation or DSB repair. Our results indicate that Ku haploinsufficiency is not a commonly observed phenomenon in human somatic cells.

2. Materials and methods

2.1. Vector construction

A *KU70* targeting vector was constructed in pMC1DT-ApA (Kurabo, Osaka, Japan) carrying a diphtheria toxin A gene cassette. Briefly, 3.6 and 4.1 kb *KU70* genomic fragments containing part of intron 2 and intron 5, respectively, were obtained by PCR using Nalm-6 genomic DNA as template. These fragments were used as 5′- and 3′-arms, respectively. The primers used were kku70-1 (5′-GTTCTTGTAGTTGGCACACCAGA-3′) and kku70-2 (5′-GATAGGCCATTGCGGCCATAGAACACCACAGCCAAGAGA-3′) for the 5′-arm, and kku70-3 (5′-GGAGACCTTGAATCACTCATTGCC-3′) and kku70-4 (5′-CCATAGGACGTTCTCATCTGAGAG-3′) for the 3′-arm. A floxed hygromycin resistance gene cassette was inserted between the 5′- and 3′-arms, thus yielding the targeting vector.

Likewise, 2.8 and 2.3 kb *KU86* (*XRCC5*) fragments were obtained by genomic PCR using primers kku86-1 (5′-GGGGACAACTTTGTATAGAAAAGTTGAGTGGTAGTTGTCTCTGAAGGGTC-3′), kku86-2 (5′-GGGGACTGCTTTTTTGTACAAACTTGCAGCTGCCTGGAAACAAAGTTCCA-3′), kku86-3 (5′-GGGGACAGCTTTCTTGTACAAAGTGGTAAGATGGATGCTTGTCTAGGCGG-3′), and kku86-4 (5′-GGGGAC AACTTTGTATAATAAAGTTGTCCATGCTCACGATTAGTGCATCC-3′). To construct a *KU86* targeting vector, the two genomic fragments and a floxed hygromycin resistance gene were assembled into a plasmid carrying a diphtheria toxin A gene cassette by using the MultiSite Gateway system (Invitrogen, Carlsbad, CA). The materials and detailed protocol for this assembly will be described elsewhere (Iiizumi et al., in preparation).

2.2. Cell culture and transfection

Nalm-6 cells were cultured in ES medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% calf serum and 50 μM 2-mercaptoethanol at 37 °C in a humidified atmosphere of 5% CO_2 in air. HCT116 cells were grown at 37 °C in McCoy's 5A medium (Gibco) supplemented with 10% FCS in a humidified atmosphere of 5% CO_2 in air. HeLa cells were maintained as previously described [24]. DNA transfection was performed as described previously [25]. Briefly, 4×10^6 cells were electroporated with 4 μg of DNA construct, and incubated for 2–3 weeks at 37 °C in agarose medium containing 0.4 mg/ml hygromycin B (Wako Pure Chemical, Osaka). Genomic DNA was isolated from drug-resistant colonies and subjected to Southern blot analysis as described previously [26].

2.3. Western blotting

Western blot analysis was carried out as described previously [27]. Anti-Ku70 and -Ku86 monoclonal antibodies were purchased from BD Biosciences (Bedford, MA). Anti-p53 monoclonal antibody (DO-1) and anti-phospho-p53 (Ser15) polyclonal antibody were purchased from Santa Cruz and Cell Signaling Technology, respectively. Levels of expression were quantified using an ATTO CS Analyzer ver2.0 (ATTO, Tokyo, Japan).

2.4. Clonogenic assays

Clonogenic survival assays were performed as previously described [26]. Briefly, exponentially growing cells were plated at 10^2–10^5 cells/dish into 60 mm bacterial dishes containing 5 ml of agarose medium. For X-ray sensitivity assays, cells were exposed to various doses of X-rays, as described [26,28]. For VP-16 sensitivity assays, cells were plated as above along with various concentrations of VP-16 (Sigma–Aldrich, St. Louis, MO). After a 2–3 weeks incubation at 37 °C, visible colonies were counted, and the percent survival was determined by comparing the number of surviving colonies to