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Lysines 3241 and 3260 of DNA-PKcs are important for genomic stability and radioresistance

Eiichiro Mori [a, b], Anthony J. Davis [a], Masatoshi Hasegawa [b], David J. Chen [a, *]

[a] Division of Molecular Radiation Biology, Department of Radiation Oncology, University of Texas Southwestern Medical Center, 2201 Inwood Rd, Dallas, TX 75390, United States
[b] Department of Radiation Oncology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

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ABSTRACT

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that plays an essential role in the repair of DNA double-strand breaks (DSBs) in the non-homologous end-joining (NHEJ) pathway. The DNA-PK holoenzyme consists of a catalytic subunit (DNA-PKcs) and DNA-binding subunit (Ku70/80, Ku). Ku is a molecular sensor for double-stranded DNA and once bound to DSB ends it recruits DNA-PKcs to the DSB site. Subsequently, DNA-PKcs is activated and heavily phosphorylated, with these phosphorylations modulating DNA-PKcs. Although phosphorylation of DNA-PKcs is well studied, other post-translational modifications of DNA-PKcs are not. In this study, we aimed to determine if acetylation of DNA-PKcs regulates DNA-PKcs-dependent DSB repair. We report that DNA-PKcs is acetylated *in vivo* and identified two putative acetylation sites, lysine residues 3241 and 3260. Mutating these sites to block potential acetylation results in increased radiosensitive, a slight decrease in DSB repair capacity as assessed by γH2AX resolution, and increased chromosomal aberrations, especially quadriradial chromosomes. Together, our results provide evidence that acetylation potentially regulates DNA-PKcs.

1. Introduction

DNA double-stranded breaks (DSBs) are deleterious DNA lesions that are primarily repaired by two pathways; non-homologous end-joining (NHEJ) and homologous recombination (HR) [1]. NHEJ is the prominent pathway responsible for repairing DSBs in human cells [2]. A central player in NHEJ is the DNA-dependent protein kinase (DNA-PK) (see reviews for more details) [3,4]. DNA-PK consists of a DNA binding subunit (Ku70/80, Ku) and a catalytic subunit (DNA-PKcs). DNA-PKcs is composed of HEAT (Huntington-elongation-A-subunit-TOR) repeats in its N-terminus, which produce a pincer-shaped structure that forms a central channel and a C-terminal region that contains the PI3 kinase domain, which is flanked by the FAT (FRAP, ATM, TRRAP) domain at its N-terminal side and by the FATC domain at its C-terminal side [5,6]. Following DSB induction, the Ku heterodimer quickly binds to the DSB ends and recruits DNA-PKcs to the break site, which is mediated by the N-terminal region of DNA-PKcs [7–9]. Upon interacting with the DSB-Ku complex, DNA-PKcs is activated. It is believed that activation of DNA-PKcs is dependent on a conformational change in both the FAT and FATC domains [4]. Once activated, DNA-PKcs phosphorylates a number of substrates with the best characterized being itself.

Following DSB formation, DNA-PKcs is heavily phosphorylated and these phosphorylations are critical for its role in DSB repair [4,10]. DNA-PKcs autophosphorylates itself and is also phosphorylated by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) protein kinases [11,12]. A large number of the phosphorylation sites are clustered in different regions of DNA-PKcs [13–16]. Two prominent clusters phosphorylated in response to DSB induction are the T2609 [13,17] and S2056 [11,18,19] phosphorylation clusters. S2056 is an autophosphorylation site [18], whereas phosphorylation of the T2609 cluster can be mediated by DNA-PKcs, ATM, or ATR. Blocking phosphorylation of DNA-PKcs at the T2609 phosphorylation cluster by mutating the serines/threonines to alanine results in blocking the release of DNA-PKcs from DNA ends, reduced DSB repair capacity, and increased radiosensitivity [17,20,21]. Blocking phosphorylation of the S2056 cluster causes increased radiosensitivity and results in increased DNA end processing, suggesting that it is required for NHEJ [19,22]. Although, regulation of DNA-PKcs through its

* Corresponding author.
E-mail address: David.Chen@UTSouthwestern.edu (D.J. Chen).

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phosphorylation has been extensively investigated, other post-translational modifications of DNA-PKcs are not well studied.

Acetylation of lysine residues is a reversible posttranslational modification, which neutralizes the positive charge of this amino acid and changes the functionality of the protein in diverse ways [23]. There are many individual reports of protein acetylation modulating diverse biological processes, suggesting that lysine acetylation has broad regulatory functions [24]. For example, it plays a key role in the regulation of gene expression through the modification of core histone tails by histone acetyltransferases (HATs) or histone deacetylases (HDACs) [25]. Lysine acetylation has also been implicated to play a role in modulating the activity of DSB repair proteins, including Ku70 [26], ATM [27], and CtIP [28]. A recent proteomic study revealed that there are at least 16 lysine residues acetylated in DNA-PKcs [29], but the biological function of DNA-PKcs acetylation is not characterized. Here, we show that DNA-PKcs is acetylated, and we identified two lysine residues (K3241 and K3260) that are potentially acetylated on DNA-PKcs. Mutating these lysine residues to block acetylation results in increased radiosensitivity and chromosomal aberrations, suggesting that these sites are important for DNA-PKcs-dependent DSB repair. Collectively, the data provide initial evidence that acetylation may modulate DNA-PKcs.

2. Materials and methods

2.1. Cell culture and transfections

Chinese hamster ovary (CHO) DNA-PKcs-deficient (V3) cell line [30] and V3 cells stably expressing YFP-tagged DNA-PKcs 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 at 37 °C in a humidified incubator with 5% CO_2. For generation of stable cell lines, cells were transfected with the linearized expression plasmid using Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer's procedures. Stable cell lines expressing YFP-tagged DNA-PKcs were maintained with 500 μg/mL of G418.

2.2. Immunofluorescence, western blotting and antibodies

Immunofluorescence was performed as described previously [31]. Nuclear extraction, immunoprecipitation and western blotting were performed as described previously [22]. Anti-γH2AX (07–164; mouse monoclonal, Millipore), anti-pS2056 (ab124918; rabbit monoclonal, abcam), anti-acetyl-lysine (SA615; rabbit polyclonal, Enzo Life Sciences) are commercially available.

2.3. Cell survival and chromosome analysis

Cell survival curves were obtained by measuring the colony forming abilities of cell populations irradiated with varying doses of irradiation as previously described [32]. Chromosome analysis was performed as described previously [33]. Briefly, exponentially growing cells were irradiated, and cultured in a presence of colcemid (1 μg/mL), starting 30 min post-irradiation for 4 hr. Mitotic cells were harvested by trypsinization and then treated with a hypotonic solution. Cells were fixed in methanol:acetic acid (3:1) and chromosomes were spread by air drying. After the slides were stained with Giemsa, chromosome aberrations were scored.

2.4. Live cell imaging and laser micro-irradiation

Live cell imaging combined with laser micro-irradiation was performed as described previously [20]. Fluorescence signal of YFP-DNA-PKcs was monitored by using an Axiovert 200 M 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. Analysis of acquired images was done as previously described [34]. Briefly, fluorescence intensity (IN) of each time point was based on pre-laser background intensity using the formula: $IN(t) = Id_t/Ib_t \times Ib_{preIR}$ [Id_t: the difference between the accumulation spot intensity and the undamaged site background intensity of each time point; Ib_t: the background intensity of each time point; Ib_{preIR}: the background intensity before irradiation. Relative fluorescence intensity (RF) was calculated using the formula: $RF(t) = (IN_t\text{-}IN_{preIR})/(IN_{max}\text{-}IN_{preIR})$ [IN_{preIR}: IN of the micro-irradiated area before laser damage; IN_{max}: the maximum IN in the micro-irradiated area of all time points]. Each data point is the average of 10 independent measurements.

2.5. Statistics

Statistical analysis was performed utilizing the student's *t*-test (paired, 1-sided). We refer to statistically significant as $p < 0.05$. Each point represents the mean ± SD of three independent experiments unless otherwise stated.

3. Results

3.1. Mutating 8 potential acetylation sites of DNA-PKcs results in increased radiosensitivity

A proteomic study identified 16 potential lysine residues acetylated in DNA-PKcs; therefore, we postulated that acetylation modulates DNA-PKcs activity [29]. To test this hypothesis, we first examined whether DNA-PKcs is acetylated *in vivo*. Immunoprecipitated DNA-PKcs from HeLa nuclear extract was probed with anti-pan-acetyl-lysine antibodies, and we found that DNA-PKcs is acetylated in normal cycling cells (Fig. 1A). Next, we aimed to identify acetylation sites that may modulate the activity of DNA-PKcs. From the 16 lysine residues identified in the proteomic study [29], we initially focused on eight lysines located around the FAT and kinase domains of DNA-PKcs, as we speculated that acetylation at these sites may affect the activity of DNA-PKcs. Approximate position of each lysine is illustrated in Fig. 1B. To examine if these lysines modulate DNA-PKcs function, we blocked potential acetylation at these sites by replacing the lysines (K) with arginines (R), hereafter termed as 8KR. The 8KR mutant was stably expressed in the DNA-PKcs-deficient Chinese hamster ovary (CHO) cell line, V3, and we assessed if mutating these putative acetylation sites affected the repair of DNA damage by monitoring cell survival to varying doses of IR. As shown in Fig. 1C, DNA-PKcs deficient V3 cells are extremely radiosensitive ($D_{10} = 1.8$ Gy). V3 cells complemented with 8KR are a moderately radiosensitive ($D_{10} = 2.8$ Gy) compared to V3 cells completed with wild-type DNA-PKcs (V3-WT) ($D_{10} = 4.0$ Gy), indicating that possible acetylation at these lysine residues plays a role in DNA-PKcs-dependent repair of DNA damage.

3.2. Mutating K3241 and K3260 of DNA-PKcs results in increased radiosensitivity

To delineate which of the eight lysine residues are required for the repair of IR-induced DNA damage, we created three groups of K-to-R mutants: K2702/2703R, K3241/3260R (termed as 2KR), K3608/3621/3638/3642R. As shown in Fig. 2A, V3 cells complemented

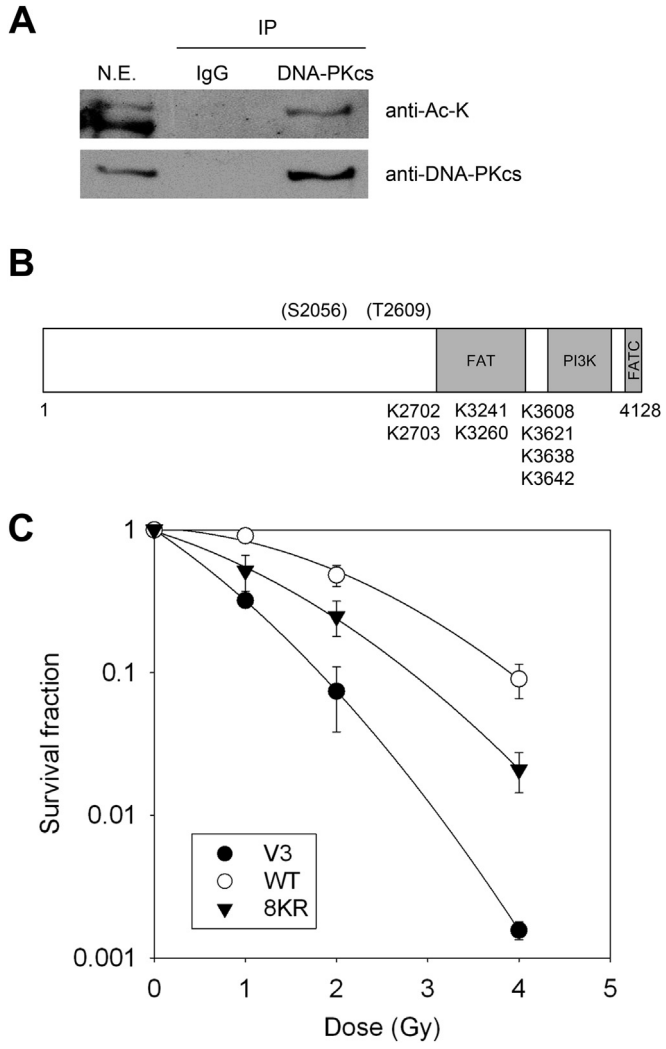

Fig. 1. DNA-PKcs is acetylated and blocking acetylation causes radiosensitivity. (A) Immunoprecipitated DNA-PKcs from HeLa nuclear extract was detected by pan-acetyl-Ab. (B) Summary of sites of K-to-R mutations in DNA-PKcs. (C) V3 cells complemented with wild-type or 8KR mutant DNA-PKcs were subjected to clonogenic survival analysis following irradiation with indicated doses.

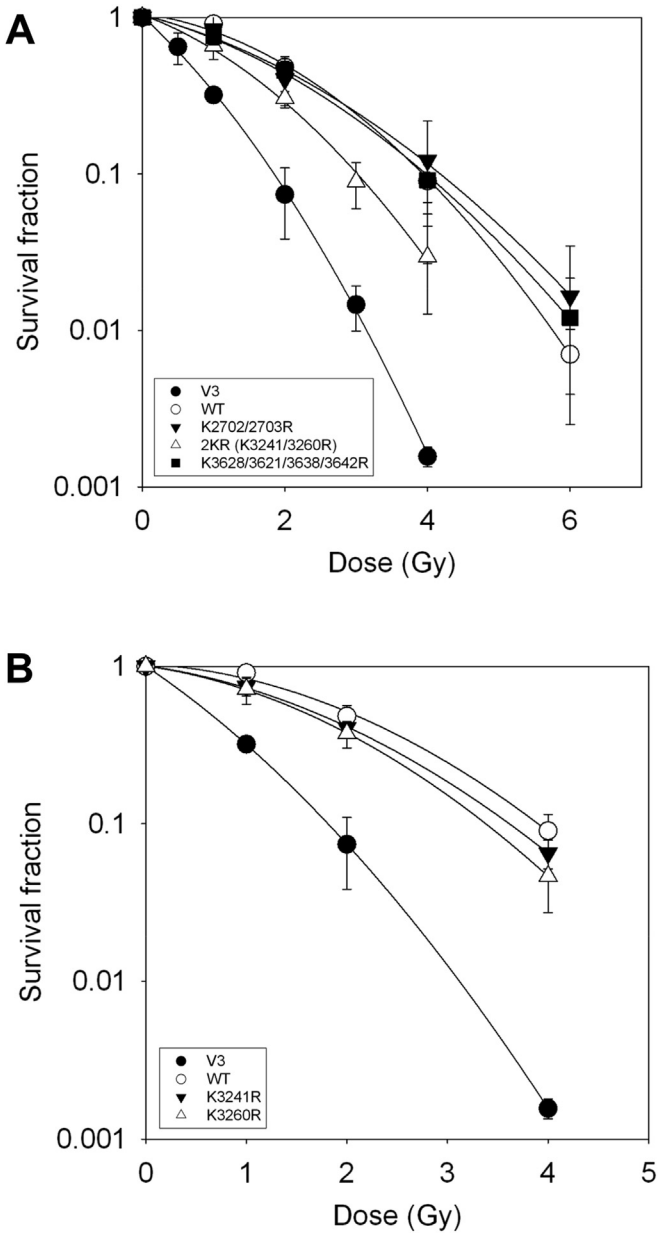

Fig. 2. K3241/K3260 have more impact among 8 acetylation sites in C-term of DNA-PKcs. (A–B) V3 cells complemented with wild-type or 2KR (K3241/3260R), K2702/2703R, K3608/3621/3638/3642R (A), or K3241R, K3260R (B) mutant DNA-PKcs were subjected to clonogenic survival analysis following irradiation with indicated doses.

with 2KR are moderately sensitive ($D_{10} = 3.2$ Gy) compared to V3-WT cell line and show similar radiosensitivity as the V3-8KR cells. On the other hand, V3 cells complemented with K2702/2703R ($D_{10} = 4.0$ Gy) or K3608/3621/3638/3642R ($D_{10} = 4.0$ Gy) showed radioresistance similar to the V3-WT cell line (Fig. 2A). V3 cells complemented with K3241R ($D_{10} = 3.6$ Gy) and K3260R ($D_{10} = 3.3$ Gy) also showed less resistance than V3-WT cell line, but not as similar as 8KR or 2KR (Fig. 2B). Collectively, the results suggest that both K3241 and K3260 play an important role in the DNA-PKcs-dependent repair of IR-generated DSBs.

Lysines 3241 and 3260 are conserved in all known DNA-PKcs homologues (Fig. 3A), suggesting that these two lysine residues may be required for DNA-PKcs function. To check if the phenotypes we observed with the K-to-R mutations on K3241 and K3260 were simply due to a general defect in growth, we examined cell proliferation in V3 cells and V3 cells complemented with WT and 2KR. As shown in Fig. 3B, 2KR did not lead to growth defects. To determine if radiosensitivity of 2KR is caused by defective DSB repair, we monitored DSB repair via IR-induced γH2AX foci resolution. As shown in Fig. 3C, absolute number of induced γH2AX foci at 30 min after IR (1 Gy) was quite different among the different cell lines (25.0 ± 2.5 for V3 cells, 22.4 ± 4.5 for WT, and 19.5 ± 2.8 for 2KR); therefore, we used relative number of γH2AX foci to the ones at 30 min after IR for our analyses. Relative remaining γH2AX foci number at 2 hr after IR was slightly higher in 2KR than WT (0.84 ± 0.11 for V3 cells, 0.59 ± 0.21 for WT, and 0.74 ± 0.22 for 2KR) (Fig. 3D). Although the difference between WT and 2KR was not significant ($p = 0.069$) at 2 hr post-IR, the trend does suggest that blocking potential acetylation at these two sites mildly impairs DSB repair.

Next, we aimed to determine if mutating these two lysines modulates the activity of DNA-PKcs. It was previously shown that impairment of either the kinase activity or clustered phosphorylation of DNA-PKcs leads to sustained retention time at laser-induced DSBs [20,35]. To check if these two residues play a role in regulating the dynamics of DNA-PKcs at DSBs, we examined the kinetics of 2KR at laser-generated DSBs. The 2KR-mutation did not

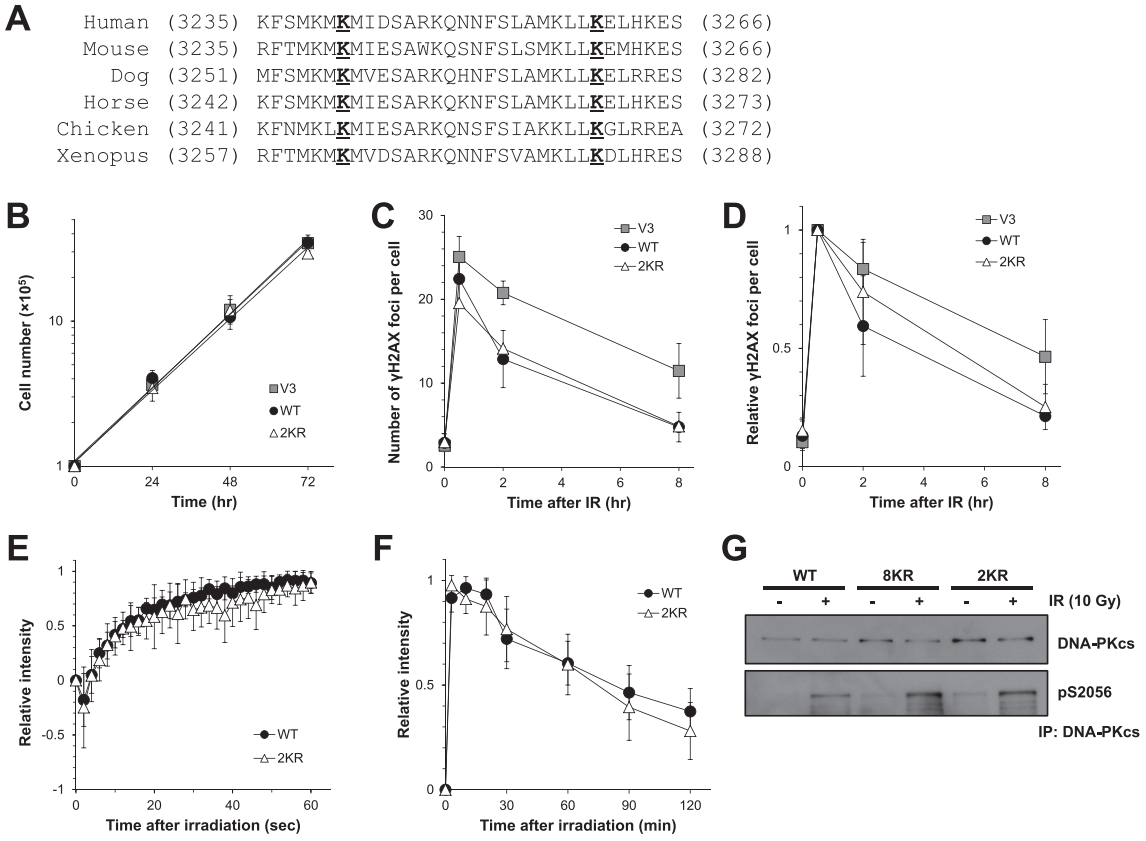

Fig. 3. Effects of mutation at K3241 and K3260 of DNA-PKcs on growth, DSB repair, recruitment/dissociation, and autophosphorylation. (A) Alignment of human DNA-PKcs K3241 and K3260 with DNA-PKcs sequences. (B) Growth curves corresponding to the indicated cell lines. (C, D) Cells were subjected to 1 Gy of γ-rays and γH2AX immunostaining. DSB repair kinetics by γH2AX foci formation was analyzed; absolute number (C) and relative number (D). (E) Initial accumulation kinetics of WT and 2KR (K3241/3260R) at laser-generated DSBs. (F) 2-hr kinetics of WT and 2KR (K3241/3260R) at laser-generated DSBs. (G) V3 cells complemented with WT, 8KR, or 2KR (K3241/3260R) DNA-PKcs were subjected to 10 Gy of γ-rays and recovered for 30 min. IR-induced autophosphorylation of DNA-PKcs was detected by anti-pS2056 antibody.

cause any defects on the recruitment and retention of DNA-PKcs at DSBs in cycling cells when assayed using live cell imaging combined with micro-irradiation (Fig. 3E and F). Next, we assessed if mutating these sites affects the kinase activity of DNA-PKcs, as they lie in the FAT domain of DNA-PKcs, which has previously been implicated to be required for DNA-PKcs activity [36–38]. Surprisingly, 8KR and 2KR have similar kinase activity as WT, as assessed via radiation induced auto-phosphorylation of DNA-PKcs at S2056 (Fig. 3G).

3.3. K3241 and K3260 of DNA-PKcs are important for genomic stability

Finally, in order to test if mutation of DNA-PKcs at K3241 and K3260 has any effect on genomic stability, we measured IR-induced chromosome aberrations. As shown in Fig. 4A, IR-induced chromosomal aberrations were significantly higher in 2KR cells (0.86 ± 0.14 per cell) compared with WT cells (0.67 ± 0.07 per cell) ($p = 0.031$). Furthermore, as shown in Fig. 4B, quadriradial

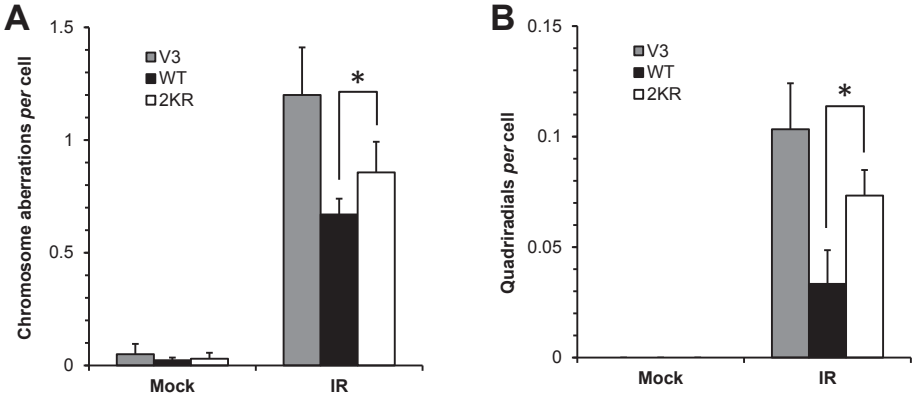

Fig. 4. Chromosome analysis of 2KR cells compared with WT and V3 cells. (A, B) Cells were subjected to 0.5 Gy of γ-rays and mitotic cells were analyzed for chromosomal aberrations; (A) total chromosome aberrations and (B) quadriradial-type chromosome aberrations (*$p < 0.05$).

chromosomes [39] were detected significantly more readily in 2KR cells compared to WT cells ($p = 0.029$). The frequency of IR-induced quadriradial chromosome aberrations was 0.033 ± 0.015 per cell in WT cells and 0.073 ± 0.012 per cell in 2KR cells.

4. Discussion

For the past decade, extensive work has been performed to identify the regulatory mechanisms governing the activity of DNA-PKcs. A number of groups have revealed that phosphorylation of DNA-PKcs is a key post-translational modification that modulates DNA-PKcs [4,10]. In this study, we aimed to provide evidence that acetylation regulates DNA-PKcs. We showed that DNA-PKcs is acetylated *in vivo* in normal cycling cells. Furthermore, blocking potential acetylation at two sites previously identified to be acetylated in a proteomic screen in the C-terminus of DNA-PKcs results in moderate radiosensitivity, a small decrease in DSB repair capacity, and increased chromosomal aberrations. This data shows that mutating the potential acetylation lysines results in attenuated DNA-PKcs dependent DSB repair.

Interacting with the Ku-DNA complex results in a conformational change in the FAT and FATC domains of DNA-PKcs, which is believed to result in the alteration of the catalytic groups and/or the ATP binding pocket of DNA-PKcs, leading to full activation of its kinase activity [36–38]. As lysines 3241 and 3260 are in the FAT domain of DNA-PKcs, we postulated that acetylation of DNA-PKcs at these sites would directly modulate the kinase activity of DNA-PKcs. However, ablating potential acetylation of K3241 and K3260 did not significantly alter the kinase activity of DNA-PKcs as monitored by autophosphorylation of DNA-PKcs. Furthermore, we did not find that mutating these sites results in a defect in the dynamics of DNA-PKcs at laser-generated DSBs. Therefore, increased radiosensitivity and reduced DSB repair capacity by mutation at these sites are not due to alterations in the two primary functions of DNA-PKcs.

As lysines 3214 and 3260 do not affect the two primary functions of DNA-PKcs, we postulate that they are required for a secondary function of DNA-PKcs. We hypothesize that they may be required for the ability of DNA-PKcs to modulate HR and/or DSB repair pathway choice. Previously, the phosphorylation status of DNA-PKcs was shown to play a role in regulating the HR pathway [40]. In addition, the role of DNA-PKcs in controlling HR pathway was reported to be dependent on the T2609 phosphorylation cluster [41,42]. Moreover, one of the pro-HR factors BRCA1 blocks autophosphorylation of DNA-PKcs at the S2056 phosphorylation cluster and promotes HR pathway [22]. DSB repair analysis by γH2AX resolution shows that there is a minor difference between 2KR and WT cells in repairing DSBs. However, chromosome analysis in cells exposed to IR showed that 2KR cells had increased chromosome aberrations compared to WT cells, in particular a significant increase in quadriradial-type aberrations was observed in the 2KR cells. The quadriradial-type of chromosome aberration is a chromatid-type aberration, which is frequently observed in cells with HR deficiency [43]. The HR pathway helps to maintain a high level of genome stability, even though immediate cell survival may be only minimally affected in cells with HR deficiency [44]. If 2KR cells have deficiency in regulating the HR pathway, the intermediate radiosensitivity and quadriradial chromosomes in 2KR cells may explain why radiosensitivity of 2KR cells are mild compared with NHEJ-deficient V3 cells.

How lysines 3241 and 3260 affect DNA-PKcs-dependent DSB repair remains unclear. It is possible that these two sites are required for the ability of DNA-PKcs to interact with a specific NHEJ/HR factor, required for a subset of NHEJ/HR, or influence the kinase activity of DNA-PKcs moderately. Furthermore, it is possible that these sites are required for DNA-PKcs function that is required for the cellular response to DSBs that is independent of DSB repair. It is also indicated that secondary replication-associated DSBs formed following exposure to IR are major substrates for IR-induced HR repair [45]. DNA-PKcs regulates the replication stress response through Chk1-Claspin pathway [46] and chromosome stability through the Chk2-BRCA1 pathway [47]. Thus, it is possible that the quadriradial chromosomes observed in 2KR cells might be arising from these secondary replication-associated DSBs after IR combined with its deficiency in appropriate control of HR pathway. Altogether, our data suggests that mutating lysines 3241 and 3260 to arginines to potentially blocking acetylation at these residues leads to a mild defect in DSB repair and decreased genomic stability. Undoubtedly, further investigation is required to elucidate the role of these lysine residues and potentially acetylation at these sites in regulating DNA-PKcs.

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