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Characteristics of DNA-binding proteins determine the biological sensitivity to high-linear energy transfer radiation

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ABSTRACT

Non-homologous end-joining (NHEJ) and homologous recombination repair (HRR), contribute to repair ionizing radiation (IR)-induced DNA double-strand breaks (DSBs). Mre11 binding to DNA is the first step for activating HRR and Ku binding to DNA is the first step for initiating NHEJ. High-linear energy transfer (LET) IR (such as high energy charged particles) killing more cells at the same dose as compared with low-LET IR (such as X or γ rays) is due to inefficient NHEJ. However, these phenomena have not been demonstrated at the animal level and the mechanism by which high-LET IR does not affect the efficiency of HRR remains unclear. In this study, we showed that although wild-type and HRR-deficient mice or DT40 cells are more sensitive to high-LET IR than to low-LET IR, NHEJ deficient mice or DT40 cells are equally sensitive to high- and low-LET IR. We also showed that Mre11 and Ku respond differently to shorter DNA fragments *in vitro* and to the DNA from high-LET irradiated cells *in vivo*. These findings provide strong evidence that the different DNA DSB binding properties of Mre11 and Ku determine the different efficiencies of HRR and NHEJ to repair high-LET radiation induced DSBs.

INTRODUCTION

DNA double-strand breaks (DSBs) induced by ionizing radiation (IR) are a severe threat to survival. There are two major pathways in mammalian cells to repair DNA DSBs: non-homologous end-joining (NHEJ) and homologous recombination repair (HRR). It has been known for several decades that high linear energy transfer (LET) IR kills more cells at the same dose compared to low-LET IR. High-LET IR is induced by high-chargeed (HZE) particles [a component in space radiation (1)], high-energy ions or by special clinical radiotherapy machines. Low-LET IR includes X or γ ray (a major component of IR from standard clinical radiotherapy machines). The more cell death induced by high-LET IR versus low-LET IR at the same dose reflects a higher relative biological effectiveness (RBE). The RBE on all kinds of quantitative effects induced by IR in X-ray exposed cells is 1. The RBE of cell killings induced by high-LET IR is ∼2–5 in cultured cells (killing ∼2–5 times the number of cells at the same dose), depending on the cell type. We and other groups recently reported that the reason high-LET IR can kill more cells than low-LET IR at the same dose is due to inefficient Ku-dependent NHEJ repair (2–4). However, it remains unclear whether this is the case *in vivo* because whole-body irradiation induces multi-system changes and results in much more complicated responses.

The mechanism explaining the phenotypes might be related to the following fact: the coordinated ionization events produced along the tracks of heavy ions generate more damage in nearby components of the DNA molecule than that of low-LET IR, thereby inducing more short DNA DSB fragments (<40 bp) at the same dose than does low-LET IR, even though high- and low-LET IR induce an equal number of DNA DSBs (4). These short DNA DSB fragments could prevent Ku from efficiently binding to the two ends of DNA at the same time and

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delay Ku-dependent NHEJ (4). Ku binding to DNA DSBs is the first step in NHEJ, which serves to recruit other essential NHEJ factors to the site of DNA DSBs. However, it remains unclear why high-LET IR does not affect HRR efficiency. Mre11 binding to DNA DSBs is the first step for activating HRR (5–7). The recently published results about DNA-binding properties of Mre11 suggest that Mre11 homodimer ($Mre11_2$) binds to the two ends of two DNA fragments (6), which might be relevant for HRR response to high-LET IR-induced DNA DSBs.

Here, we showed that although high-LET IR can efficiently kill more wild type and HRR-deficient mice or cells compared to low-LET IR, it could not kill more Ku-dependent NHEJ-deficient mice and cells. These results demonstrate for the first time at the animal level that high-LET radiation induced high RBE is due to inefficient Ku-dependent NHEJ. In addition, we showed the different binding properties of Mre11 and Ku to DNA DSB *in vitro* and *in vivo*. Based on these results, we believe that the different DNA-binding properties of Mre11 and Ku determine the different effects of high-LET radiation on HRR and NHEJ.

MATERIALS AND METHODS

Mouse strain, cell lines and irradiation

$Rad54^{-/-}$ (HRR deficient), $Ku70^{-/-}$ or DNA-$PKcs^{-/-}$ (NHEJ deficient) mice were generated as described previously (8–10). Wild-type control mice were ordered from Jackson Laboratory, Bar Harbor ME, USA. These mice (~6 weeks old, three male and three female for each group) were exposed to IR and observed daily for 30 days. All the mice experiments were performed according to the approved animal protocol. Chicken DT40 cell lines: wild type, $Ku70^{-/-}$, $Rad54^{-/-}$ and conditional $Rad51^{-/-}$ cells were generated and maintained as described previously (11,12). DT40 cell sensitivity to radiation was determined by the loss of colony-forming ability in the medium containing 1.5% (w/v) methylcellulose (Sigma-Aldrich, St Louis MO, USA) as described previously (11,13). Tetracycline (Tet) 20 ng/ml was added to the culture of the conditional $Rad51^{-/-}$ cells for 12 h before the cells were exposed to radiation. This treatment resulted in knocking down 90% of Rad51 in the cell line (12). After IR, the cells were transferred to new dishes with fresh medium without Tet for colonies forming according to the previously reported methods (13). The transformed mouse embryo fibroblasts cells were maintained as described previously (4). High-LET IR was carried out using the alternating-gradient synchrotron (AGS, Fe ions, 1 GeV/amu, LET: 150 keV/μm, Range in Water: 27 cm, Radiation area: 20 × 20 cm, uniformity: ±25%) at the Brookhaven National Laboratory. Low-LET IR was carried out using an X-ray machine (X-RAD 320, N. Branford 320 kV, 10 mA, the filtration with 2-mm aluminum for cells and the filter with 1.5 mm aluminum, 0.8-mm tin and 0.25-mm copper for mice) in our laboratory. The dose rates for both high-LET IR and low-LET IR were about 1 Gy/min.

Electrophoretic mobility shift assay

Ku protein was purified as described previously (14). Mre11 protein was a gift from Dr. Tainer (6). Double-strand DNA (dsDNA) end binding of the proteins was assessed by EMSA as described previously (4). Equal molecules of DNA (0.4 nM) of various lengths (30 and 75 bp) were 5′-end labeled with [γ-^{32}P]-ATP and polynucleotide kinase (Fisher Scientific, Pittsburgh, PA, USA). The non-labeled free [γ-^{32}P]-ATP in the sample was removed by using the NucAway Spin Columns (Ambion Inc.). Electrophoretic mobility shift assay (EMSA) was performed by incubating the purified 0.0125–0.05 μM Ku protein or 0.5–5 μM Mre11 protein with [γ-^{32}P]-ATP labeled DNA (30 and 75 bp) in 10 μl of reaction mixture (20 mM Tris–HCl (pH 7.8), 2 mM dithiothreitol, 80 μg/ml bovine serum albumin and 100 mM KCl) at room temperature for 30 min. Gel loading buffer [2.5 μl, 250 mM Tris–HCl (pH 7.5), 0.2% bromphenol blue and 40% glycerol] was added to the reaction. The products were then separated on a 5% nondenaturing polyacrylamide gel (130 V) in Tris borate/EDTA buffer at 4°C for 2 h. The signals of the labeled DNA fragments were detected by a PhosphorImager (GE, Portland, OR, USA) with ImageQuant software (GE, Portland, OR, USA).

Detecting Ku or Mre11 in chromatin DNA–protein complexes

The experimental procedure was similar as described previously (15). Briefly, cells were immediately collected after IR. A portion of the cells was used for detecting the levels of Ku or Mre11 in the whole cell lyses (as internal control), which were made with RIPA buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1 μg/ml each aprotinin, leupeptin and pepstatin; 1 mM Na_3VO_4; 1 mM NaF). The remaining portion of the cells was used to prepare cytoplasmic and nuclear extracts by using the NE-PER^{TM} kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. After the cytoplasmic and nuclear extracts were removed, the remained chromatin pellets were sonicated in RIPA buffer. The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Ku 70 antibody (sc-1486, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Mre11 (#4895, Cell Signaling Technology, Danvers, MA, USA) were used in this study.

Detecting DNA in the Mre11–DNA complex

The experimental procedure for detecting DNA in Ku complexes was similar as described previously (15): (i) cross linking DNA–protein from 10^7 cells; (ii) sonicating to break DNA into ~20–1000 bp; (iii) immunoprecipitating (IP) the rest of the sample with Ku70 antibody and taking 10 μl of such sample for western blot detecting Ku level as internal control; (iv) labeling the rest of the sample with γ-^{32}P ATP; (v) digesting the complex with

protease; (vi) running gel and detecting the DNA signal. The experimental procedure for detecting DNA from the Mre11 complex has two different steps. One was to use Mre11 antibodies in IP and the other was to sonicate the sample one more time before labeling. The purpose of the second sonication is to free DNA ends from Mre11 binding that occurred during IP process. The DNA signals were detected and analyzed by a PhosphorImager as described above.

RESULTS AND DISCUSSION

High-LET IR-induced high RBE value on mice survival depends on the intact Ku-dependent NHEJ

Previously, we and others reported that high RBE values on survival for high-LET irradiated cells depends on the intact Ku-dependent NHEJ (2–4), suggesting that high-LET IR induced high RBE on cell survival is due to the inefficient NHEJ. However, it remains unclear whether the situation is the same in the context of the whole body of a vertebrate animal because whole-body irradiation induces changes in multi-system and results in much more complicated responses. Therefore, we compared the survival rates of different repair pathway deficient mice at one month following high- or low-LET IR. The results showed that although all wild-type mice survived at one month following 8 Gy of low-LET radiation, all wild-type mice died within one month following 8 Gy of high-LET radiation (Figure 1A). Rad54$^{-/-}$ mice showed a similar result but with a shorter survival time when compared with wild-type mice following 8 Gy of high-LET radiation (Figure 1B). The fact that Rad54$^{-/-}$ mice were not much more sensitive to high-LET IR than wild mice might be due to that Rad54$^{-/-}$ mice are not completely deficient in HRR and other HRR factors might complement the HRR function of Rad54 in the mice. These results clearly indicate that wild-type and HRR-deficient (or partially deficient) mice are more sensitive to high-LET IR than to low-LET IR. Interestingly, although all the NHEJ-deficient mice, including DNA-PK$^{-/-}$ and Ku70$^{-/-}$ mice, died within 10 days following 4 Gy of low-LET IR, they were not any more sensitive to high-LET IR, resulting in the same sensitivity of these mice to low- or high-LET IR (Figure 1C and D). We chose 4 Gy for the NHEJ-deficient mice because some of the mice still survived at one month following 3 Gy of radiation. These results exclude the possibility that overdose hides the different sensitivity of the mice to low- or high-LET IR. NHEJ-deficient mice combined with HRR deficiency dramatically increased their sensitivity to IR (16,17). If in addition to inefficiency of the Ku-dependent NHEJ, high-LET IR also affects HRR, the NHEJ-deficient mice, including DNA-PK$^{-/-}$ and Ku70$^{-/-}$ mice, should be more sensitive to high-LET IR. Therefore, these *in vivo* results provide for the first time direct evidence that high-LET IR-induced high RBE depends on the intact Ku-dependent NHEJ. These results also indicate that high-LET IR affects only the Ku-dependent NHEJ but not HRR when compared with low-LET IR. Additional evidence by using DT40 cell lines with different genetic backgrounds supports this conclusion: Ku-deficient cells showed the exact same sensitivity to low-LET or high-LET IR, although wild-type cells were more sensitive to high-LET IR than low-LET IR and HRR-deficient cell lines (Rad54$^{-/-}$ and Tet-induced Rad51 knocked down cells) were even more sensitive to high-LET IR than to low-LET IR due to the inefficient NHEJ induced by high-LET IR (Figure 2). These results also confirm that high-LET IR-induced high RBE is not due to the inefficient HRR. If high-LET also affects the efficiency of HRR, HRR-deficient mice or cells should be less sensitive to high-LET IR than their wild-type counterparts.

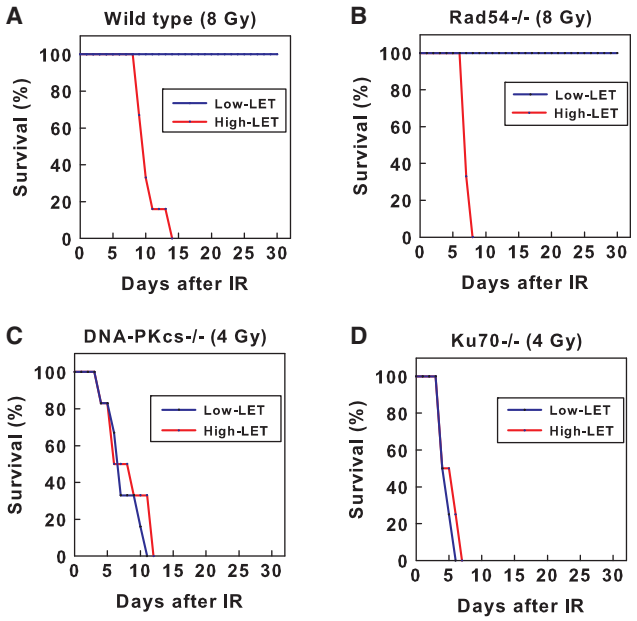

Figure 1. Sensitivity of mice to high-LET or low-LET radiation. Each irradiated group had six mice. Non-irradiated controls were two mice for each type genotype. (**A**) Surviving wild-type mice after 8 Gy high-LET or low-LET radiation. (**B**) Surviving Rad54$^{-/-}$ mice after 8 Gy high-LET or low-LET radiation. (**C**) Surviving DNA-PK$^{-/-}$ mice after 4 Gy high-LET or low-LET radiation. (**D**) Surviving Ku70$^{-/-}$ mice after 4 Gy high-LET or low-LET radiation.

Short linear DNA fragments (<40 bp) do not affect the binding efficiency of Mre11

After confirming that high-LET IR only affects the efficiency of the Ku-dependent NHEJ, the next question is why high-LET radiation does not affect HRR. Previously, we showed that high-LET IR induced more short fragments of linear DNA (<40 bp) than low-LET IR (4). We believe that the small DNA fragments are the key factor for explaining the mechanism. One round of dsDNA is ~80–100 bp and 2.5 rounds of dsDNA for one nucleosome is ~200–240 bp. High-LET IR with densely deposited energy could cause multiple damages within the nucleosome, and generate small fragments of linear DNA (<40 bp). It is known that small fragments of dsDNA affect the binding efficiency of Ku (4,18) and thereby could result in inefficient NHEJ and more cell

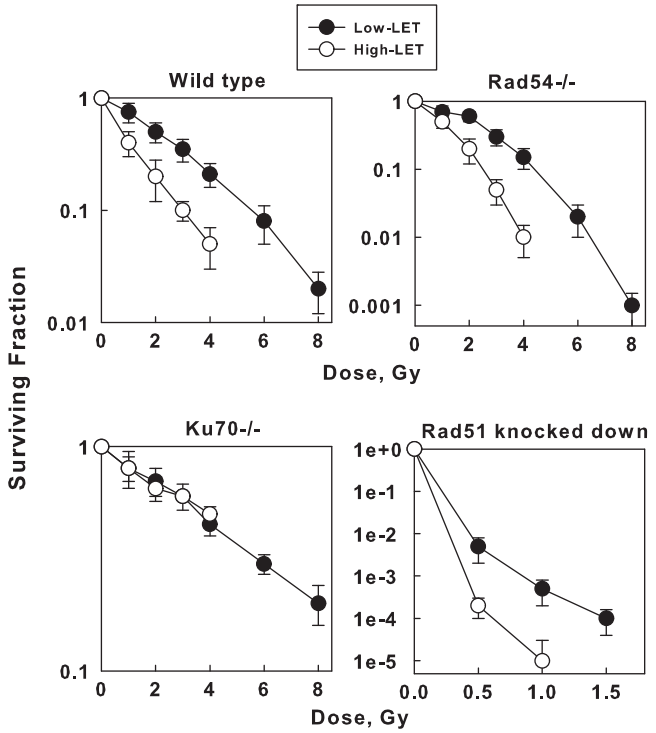

Figure 2. Comparison of the sensitivity of different repair deficient DT40 cell lines to high-LET IR or low-LET IR induced cell killing. The cells were irradiated with either high- or low-LET IR. The sensitivity of these cells to IR was then determined in a clonogenic assay with growth medium containing 1.5% (w/v) methylcellulose as described previously (11,13). Tetracycline (Tet) 20 ng/ml was added to the culture of the conditional Rad51$^{-/-}$ cells for 12 h before the cells were exposed to radiation. This treatment resulted in the cells left with ~10% of original Rad51 (12). After IR, the cells were transferred to new dishes with fresh medium without Tet for colonies forming. Two replica samples were prepared for each dose and were incubated for 1–2 weeks to allow colony formation. The data are from three to four independent experiments and are presented as means and standard error.

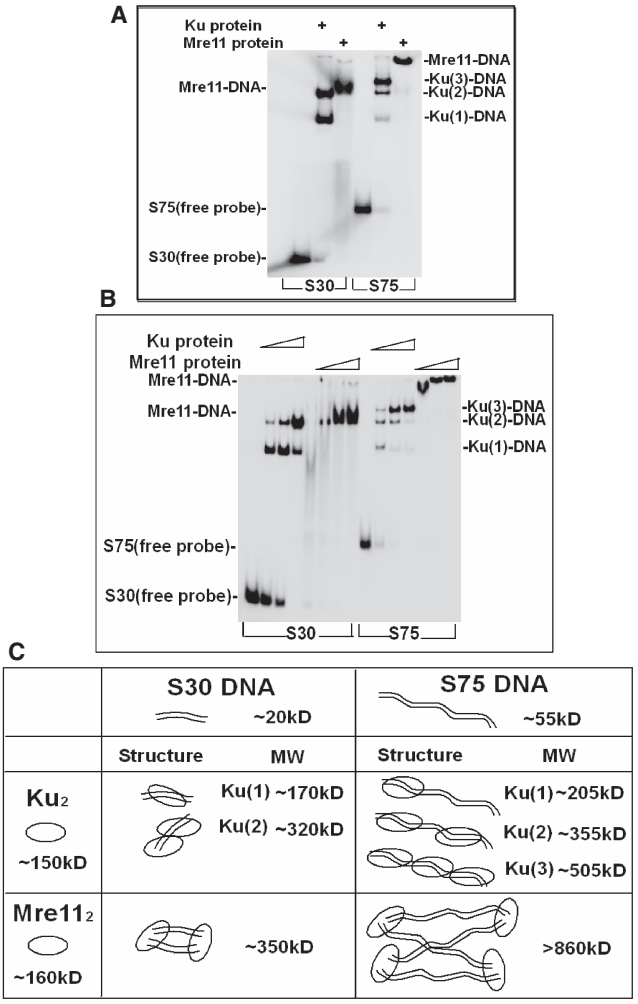

Figure 3. EMSA showing the efficiencies of Ku and Mre11 binding to small fragments of dsDNA. DNA-binding experiments were carried out with ^{32}P-labeled blunt ends of dsDNA fragments and purified Ku or Mre11 as described in 'Materials and Methods' section. (**A**) Signals of Ku or Mre11 bound to different size of dsDNA fragments. Ku(1) DNA represents Ku–DNA complex with one Ku molecule binding to one end of each dsDNA fragment, Ku(2) DNA represents Ku–DNA complex with two Ku molecules binding to the dsDNA fragment and Ku(3) with three Ku molecules binding to the dsDNA fragment. (**B**) Signals of Ku or Mre11 at different concentrations bound to dsDNA fragments. (**C**) Calculation of the molecular weight (MW) of possible structure of the protein–DNA complexes.

death *in vitro* and *in vivo*. Therefore, if the small fragments of dsDNA are the key factor affecting the efficiency of Ku-dependent NHEJ, they should be the same key factor to explain why high-LET IR does not affect HRR. Mre11 binding to DNA DSB is the initial step for HRR (6,7), and the recently published paper on the property of Mre11 binding to DNA (6) provides us with valuable information to explain the different responses of NHEJ and HRR to high-LET IR. To test whether such small fragments of dsDNA affect the DNA binding efficiency of Ku influent Mre11 binding efficiency, we compared the efficiency of Mre11 binding to dsDNA fragments with 30 and 75 bp, respectively. The results showed that although most Ku binding signals for 30 bp of dsDNA were from one Ku molecule–DNA complex (a portion of them were from two Ku molecule–DNA complexes), most Ku binding signals for 75 bp of dsDNA were from two or three Ku molecules–DNA complexes (Figure 3A). These results confirmed that the efficiency of Ku binding to DNA is affected by the DNA length. When the length of the fragments is <40 bp, the fragments prevent Ku from efficiently binding to the two ends of the DNA at the same time (4). Contrary to Ku, Mre11 did not show any apparent different change in the binding efficiency for the different sizes of dsDNA fragments (Figure 3A). The results from titrated amounts of proteins (from unsaturated to saturated amounts) in the DNA-binding experiment excluded the possibility that the different results for Ku and Mre11 were derived from less- or overloaded protein (Figure 3B). These results indicate that small fragments of dsDNA (<40 bp) that affect the DNA binding efficiency of Ku do not affect the binding efficiency of Mre11.

Based on the information about the binding properties of Ku and Mre11, we carefully calculated the size of the complex and expected the components of the complex in the EMSA (Figure 3C). Ku70 and Ku80 as a heterodimer form a tunnel-like structure and wraps DNA strands (19). One such heterodimer requires at least $\geqslant$20 bp of DNA (18). The 75-nt DNA fragment (S75) could hold up to three heterodimers (**Ku(3)**) (4,20) (Figure 3). However, when the DNA fragment is $<$40 bp (30 bp, S30), it could only hold one (**Ku(1)**) or two heterodimers (**Ku(2)**) (4,20) (Figure 3). Mre11 is different from Ku: Two Mre11 molecules form the homodimer head ($Mre11_2$) that catches two ends of DNA fragments through the minor grooves at the same time (6), and the homodimer head requires fewer nucleotides for binding than Ku heterodimer. It might be explained as follows: the structures of the Mre11-S30- and the Mre11-S75–DNA complex: when $Mre11_2$ efficiently hold two ends of the DNA fragments, this complex forms an acute angle for the two DNA fragments. Such angle results in other two ends of the short fragments (S30) closing each other, which are easily bound by an additional Mre11 homodimer head ($Mre11_2$) (Figure 3C). However, such angle has little effects on other ends of the long DNA fragments (S75), which are freely paired by the additional Mre11 homodimer heads (Figure 3C). These *in vitro* results partially reflect the different DNA binding property of Ku and Mre11. However, it should be noticed that Ku and Mre11 binding to DNA in mammalian cells are more complicated due to the chromatin structure.

High-LET IR does not affect the efficiency of Mre11 binding to chromatin DNA when compared with low-LET IR

To clarify whether the *in vitro* binding property of Mre11 could explain *in vivo* why high-LET IR does not affect HRR, we examined the chromatin DNA-binding efficiency of Mre11 in low- or high-LET irradiated cells. Mre11 did not show any changes in its levels in the protein–chromatin DNA complex from low-LET irradiated cells or from high-LET irradiated cells (Figure 4A). Differently, the levels of Ku in the protein–chromatin DNA complex from the low-LET irradiated cells were greater than the levels in the protein–chromatin DNA complex from high-LET irradiated cells (Figure 4A). These results suggest that although the DNA DSBs induced by high-LET IR when compared with those induced by low-LET IR are less efficiently bound by Ku, they are still bound in the same efficiently by Mre11. To confirm the hypothesis, we further examined the DNA levels in the Mre11–DNA complex from high- or low-LET irradiated cells.

When we initially tried to use the same protocol (detecting the DNA signals from the Ku–DNA complex) to detect the DNA signals from the Mre11–DNA complex, we could not obtain any labeled signals until we modified the protocol by sonicating the IP complex before doing the end labeling (see 'Materials and Methods' section). It might be that Mre11 in the samples during the IP process efficiently bound to the free ends of DNA that generated from the first sonication, which blocked the DNA end labeling with γ-^{32}P ATP. These results also reflect the different binding properties of Mre11 and Ku. The labeling results showed that the amount of DNA in the Ku–DNA complex from high-LET irradiated cells was clearly less than the amount from low-LET-irradiated cells. However, the amount of DNA in the Mre11–DNA complex from low-LET-irradiated cells was similar to that from high-LET-irradiated cells (Figure 4B). These results confirm that the quality of DNA DSBs in high-LET-irradiated cells is different from that in low-LET-irradiated cells, indicating that DSBs in high-LET-irradiated cells when compared with low-LET-irradiated cells are less efficiently bound by Ku, but are equally efficiently bound by Mre11.

Taken together, our results demonstrate for the first time *in vivo* that high-LET IR induced high RBE value is due to the inefficient Ku-dependent NHEJ, which can be due to inefficient Ku DNA binding. Based on these results, we formed a model: the DNA-binding property of Mre11 might explain why high-LET IR does not affect HRR efficiency (Figure 5). We believe that these results constitute valuable information to space radiation protection and clinical radiotherapy with the high-energy particle machines.

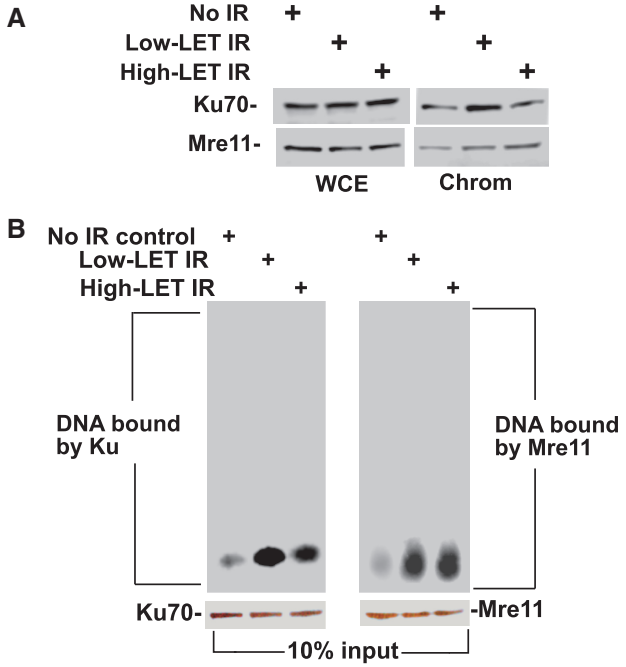

Figure 4. Comparison of Ku or Mre11 levels in the protein–DNA complex and comparison of DNA levels in the Ku–DNA or Mre11–DNA complexes from different samples. (**A**) Ku or Mre11 levels in the chromatin-bound fraction from high- or low-LET-irradiated cells. WCE: represents the signals from whole cell lyses, which was used as the internal loading control. Chrom: represents the signals from chromatin-bound fraction. Similar results were obtained from two independent experiments. (**B**) The DNA levels in the Ku–DNA or Mre11–DNA complex from high- or low-LET-irradiated cells. One-tenth of the IP samples were used as the internal loading control. Similar results were obtained from two independent experiments.

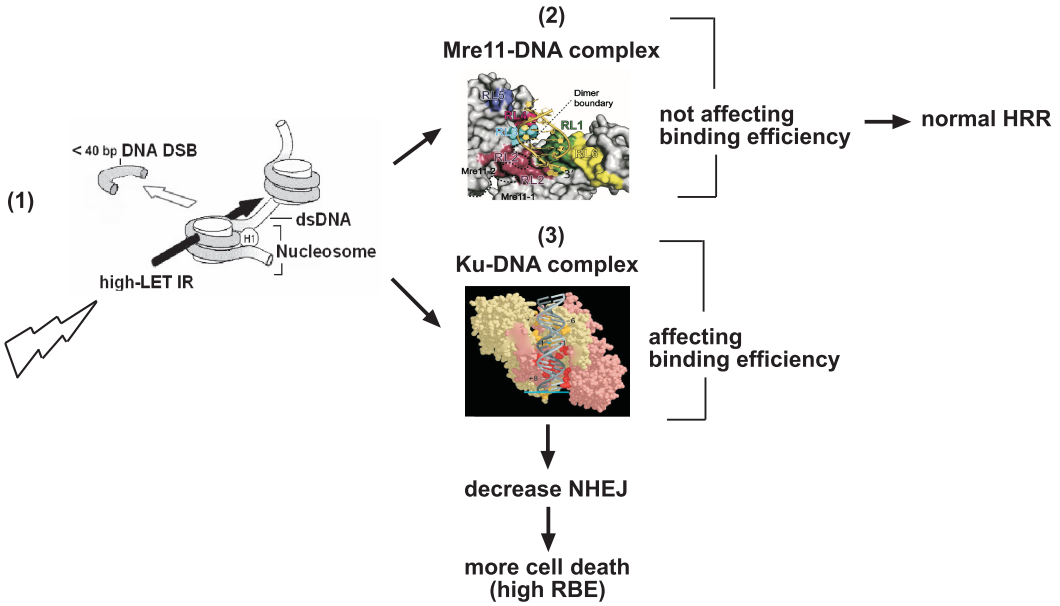

Figure 5. A model to explain the different effects of high-LET IR on NHEJ and HRR. (**1**) When cells are exposed to high-LET IR, the coordinated ionization events produced along the tracks of heavy ions generate more damage in nearby components of the DNA molecule than that of low-LET IR, which generates short fragments of linear DNA within a size range of < 40 bp. (**2**) These short fragments of linear DNA in the high-LET-irradiated cells do not affect MRE11 binding efficiency, which results in the same efficiency of HRR in high-LET or low-LET-irradiated cells. (The Mre11 protein–DNA structure is cited from ref. 6 and permission was obtained from Dr. Tainer and the Cell.) (**3**) These short fragments of linear DNA do affect Ku binding efficiency and therefore affect NHEJ, which results in more cell death and is reflected as high RBE. (The Ku protein–DNA structure is cited from ref. 19 and permission was obtained from Dr. Goldberg and the Nature.).

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