Radiation-induced XRCC4 Association with Chromatin DNA Analyzed by Biochemical Fractionation

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DNA double-strand break (DSB) repair/Non-homologous end-joining (NHEJ)/Chromatin/XRCC4/DNA-dependent protein kinase (DNA-PK).

XRCC4, in association with DNA ligase IV, is thought to play a critical role in the ligation of two DNA ends in DNA double-strand break (DSB) repair through non-homologous end-joining (NHEJ) pathway. In the present study, we captured radiation-induced chromatin-recruitment of XRCC4 by biochemical fractionation using detergent Nonidet P-40. A subpopulation of XRCC4 changed into a form that is resistant to the extraction with 0.5% Nonidet P-40-containing buffer after irradiation. This form of XRCC4 was liberated by micrococcal nuclease treatment, indicating that it had been tethered to chromatin DNA. This chromatin-recruitment of XRCC4 could be seen immediately (< 0.1 hr) after irradiation and remained up to 4 hr after 20 Gy irradiation. It was seen even after irradiation of small doses, i.e., 2 Gy, but the residence of XRCC4 on chromatin was very transient after 2 Gy irradiation, returning to near normal level in 0.2–0.5 hr after irradiation. The chromatin-bound XRCC4 represented only ~1% of total XRCC4 molecules even after 20 Gy irradiation and the quantitative analysis using purified protein as the reference suggested that only a few XRCC4-DNA ligase IV complexes were recruited to each DNA end. We further show that the chromatin-recruitment of XRCC4 was not attenuated by wortmannin, an inhibitor of DNA-PK, or siRNA-mediated knockdown of the DNA-PK catalytic subunit (DNA-PKcs), indicating that this process does not require DNA-PK, or siRNA-mediated knockdown of the DNA-PK catalytic subunit (DNA-PKcs), indicating that this process does not require DNA-PKcs. These results would provide us with useful experimental tools and important insights to understand the DNA repair process through NHEJ pathway.

INTRODUCTION

DNA double-strand breaks (DSBs) are generated externally, by ionizing radiation or radiomimetic drugs, or spontaneously, by oxidative stresses or replication errors, and, unless repaired properly, may lead to cell killing or tumorigenesis. Eukaryotic cells have two major pathways to repair DNA double-strand breaks (DSBs): non-homologous end-joining (NHEJ) and homologous recombination (HR).

One of the key players in NHEJ, especially in vertebrates, is DNA-dependent protein kinase (DNA-PK), composed of the catalytic subunit (DNA-PKcs) and heterodimeric Ku protein (Ku86 and Ku70). Because of its striking property to be activated upon binding to the ends of double-stranded DNA, DNA-PK is thought to act as the sensor to recognize DSBs, initiating NHEJ and, possibly, other DNA damage responses. Another core component of NHEJ pathway is the complex of XRCC4 and DNA ligase IV, which is thought to join the DSBs finally. Recently, a new essential factor of NHEJ, named XLF or Cernunnos, which is associated with XRCC4-DNA ligase IV complex, was identified. There are several lines of evidence in vitro indicating that DNA-PKcs and/or Ku directly associate with XRCC4-DNA ligase IV and facilitate its recruitment to DNA ends. However, it remains to be clarified how these proteins are recruited to DSB sites and assembled into repair machinery, especially in living cells.

Many proteins in the homologous recombination pathway, e.g., Nbs1-Mre11-Rad50, BRCA1 and Rad51, exhibit local accumulation after DSB induction, forming microscopically visible structures, termed ionizing radiation-induced foci (IRIF). Such change in the localization of HR proteins has been observed also in partial volume irradiation and laser micro-irradiation experiments. As the distribution of these proteins after irradiation, at least partially, overlapped with irradiated area or DSBs, visualized by DNA end labeling or immunofluorescence analysis of γ-H2AX, these phenomena are believed to reflect the accumulation of these proteins around DSB sites. In the case of NHEJ proteins, however, IRIF has been observed only for autophosphorylated form of...
DNA-PKcs. The failure or difficulty to detect IRIF of NHEJ proteins might be attributable to several reasons, which are not mutually exclusive: (i) only a very small number of molecules might be recruited to each DSB and, thereby, do not appear as foci. (ii) NHEJ proteins, especially Ku and DNA-PKcs, are abundant. Therefore, even if some NHEJ molecules really moved to DSB sites, their overall distribution would not change to a discernable extent. (iii) As NHEJ is a very rapid reaction, healing most of DSBs within minutes after irradiation, the association of NHEJ enzymes with DSB might be very transient and, thereby, difficult to be captured. Recently, several studies using laser micro-irradiation demonstrated the accumulation of NHEJ molecules in irradiated area but local dose may be high.

Another approach to examine the association of DNA repair proteins with damaged DNA is sequential extraction with increasing concentration of detergent or salt. This approach has been used to demonstrate the recruitment and retention of ATM and NbsI/Mre11/Rad50 complex to DSB site. Using this approach, the present study detected and analyzed the association of XRCC4 with chromatin DNA, which was induced by ionizing radiation. Drouet et al demonstrated DNA damage-induced mobilization of XRCC4-DNA ligase IV as well as DNA-PKcs and Ku using similar approach but there are several differences in the obtained results and conclusion, as shown below. The present study would provide us with useful experimental tools and important insights to understand the DNA repair process through NHEJ pathway.

MATERIALS AND METHODS

Cells
Murine leukemia L5178Y-derived, XRCC4 deficient cell line M10 was obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) with the permission of Dr. Koki Sato (Kinki University). The cells were cultured in RPMI1640 medium with the permission of Dr. Koki Sato (Kinki University). The cells were cultured in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C in humidified atmosphere containing 5% CO2. Human T cell leukemia MOLT-4 cells have been routinely used in our laboratory.

Full-length XRCC4 cDNA was obtained by polymerase chain reaction (PCR) from the cDNA pool of MOLT-4 cells and inserted into p3xFLAG-CMV10 vector (Sigma-Aldrich, St. Louis, MO, USA) using EcoRI and Bg/II (Takara, Otsu, Shiga, Japan). This XRCC4-expressing vector or empty vector was introduced into the cells by electroporation using Gene Pulser II (BIO-RAD Laboratories, Hercules, CA, USA). After selection by culture in the presence of 0.8 mg/ml G418, stable clones, named M10-XRCC4 and M10-CMV, were obtained, which were used for further analyses.

To knock down the expression of DNA-PKcs by siRNA, we used pSilencer 2.1-U6 hygro vector kit (Ambion, Austin, TX, USA). While three insert constructs have been tested, the most effective one was that corresponding to 7304–7324 of murine DNA-PKcs mRNA. The sequence of the duplex insert oligonucleotides are 5’-GAT CCC AGG TGT TTG TGG ATA TAG TTT CAA GAG AAC TAT ATC ACA ACA CAC CCT TTG TGG GAA A-3’ and 5’-AGC TTT TCC AAA AAA AGG TGT TTG TGG ATA TAG TGC TCT TGA AAC TAT ATC CAA ACA CAC CTC G-3’. The vector targeting DNA-PKcs or GFP, included in the kit as a control, was introduced into M10-XRCC4 cells by electroporation. Stable transfectants were selected by 0.4 mg/ml hygromycin and a clone, expressing the lowest amount of DNA-PKcs but XRCC4 at similar level to GFP control, was used for the analysis.

Cells were irradiated using either 60Co γ-ray source or X-ray generator HF-350C (Shimadzu-Pantak; Kyoto, Japan), operated at 200 kV and 20 mA with filters of 0.5 mm Cu and 1.0 mm Al. Where indicated, wortmannin (Sigma-Aldrich) was added at the concentration of 10 μM. For stock, wortmannin was dissolved in dimethyl sulfoxide at 50 mM and kept at −20°C. DNA-PK Inhibitor II and ATM Kinase Inhibitor were dissolved in dimethyl sulfoxide at 5 mM and kept at −20°C.

Analyses of radiosensitivity and proliferation rate
The radiosensitivity of M10-CMV and M10-XRCC4 was assessed in terms of their colony forming ability in 0.16% agarose (SeaKem GTG Agarose:Cambrex Bio Sciences, Rockland, ME, USA). The surviving fraction (S.F.) was normalized to unirradiated control and fitted computationally to the equation: S.F. = exp(−αD−βD2), where D is the X-ray dose. Cell proliferation rate was measured by counting the cells with an interval of approximately 12 hr using Coulter Particle Counter (Beckman Coulter, Fullerton, CA, USA).

Cell fractionation
The fractionation procedure is schematically shown in Fig. 1. In principle, it followed the protocol published by Andegeko et al. with some modifications. Typically, 107 cells, either irradiated or unirradiated, were harvested and rinsed twice with ice-cold phosphate-buffered saline (without magnesium and calcium). The cell pellet was suspended in 150 μl of buffer A (50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1/100 volume each of protease inhibitor cocktail for animal cells (Nacalai Tesque), phosphatase inhibitor cocktail I and II (Sigma-Aldrich)) with 0.2% Nonidet P-40. After standing on ice for 5 min, the suspension was centrifuged at 1,000 xg for 5 min and the supernatant was recovered as F-I. The cell pellet, denoted P-I, was resuspended in 150 μl of the same buffer and immediately centrifuged at 1,000 xg for 5 min. The supernatant of this step was recovered as F-II. The cell pellet, denoted P-II, was then resuspended in 150 μl of buffer A with 0.5% Nonidet P-40. After standing on ice for 40 min, the suspension was centri-
fuged at 16,000 xg for 5 min and the supernatant was recovered as F-III. The resultant pellet, denoted P-III, was suspended in equivalent volume of 2xSDS-PAGE loading buffer and heated in boiling water for 5 min. After centrifugation at 16,000 xg for 5 min, the supernatant was recovered as F-IV.

For the nuclease treatment, P-III was resuspended in the equivalent volume of MN buffer (10 mM HEPES-NaOH (pH 7.4), 10 mM KCl, 0.5 mM MgCl₂, 2 mM CaCl₂) supplemented with micrococcal nuclease (Worthington Biochemical, Lakewood, NJ, USA) at the dose of 1,000 U/10⁷ cells. After incubation on ice for 1 hr, the reaction was terminated by the addition of EDTA to the final concentration of 5 mM and centrifuged at 16,000 xg for 5 min. The supernatant was recovered as F-V.

For the phosphatase treatment, P-III was suspended in the reaction buffer with λ protein phosphatase (New England Biolabs, Ipswich, MA, USA) at the dose of 800 U/10⁷ cells and incubated for 30 min at 30°C. The reaction was stopped by the addition of 2xSDS-PAGE loading buffer and heated in boiling water for 5 min. After centrifugation at 16,000 xg for 5 min, the supernatant was analyzed by Western blotting as described below.
Antibodies and Western blotting

Anti-XRCC4 rabbit polyclonal antibody was generated against full-length human XRCC4, which had been expressed in E. coli. Anti-FLAG monoclonal antibody M2, conjugated with horseradish peroxidase was purchased from Sigma-Aldrich. For the detection of DNA-PKcs, a cocktail of three monoclonal antibodies was used (Ab-4, Chemicon International, Fremont, CA, USA). Anti-Ku86 rabbit polyclonal antibody was generated in our earlier studies (and references therein). For the loading control, anti-histone H2AX rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) and anti-PCNA mouse monoclonal antibody (Ab-1, Thermo Fisher Scientific, Fremont, CA, USA) were used. General procedures of Western blotting followed our earlier publications, with minor modifications. For quantification, chemiluminescent light was captured by ImageQuant 350 (GE Healthcare, Buckinghamshire, UK) and ImageQuant TL software. Human XRCC4-DNA ligase IV complex, purchased from Trevigen (Gaithersburg, MD, USA), was used as the standard to obtain the absolute number of XRCC4 molecules in the cell.

RESULTS

Establishment of XRCC4-reconstituted cell line

Murine leukemia L5178Y-derived M10 lacks endoge-
neous XRCC4 due to the mutation and allelic loss. We introduced a vector encoding human XRCC4 cDNA with amino-terminal 3xFLAG tag and the control vector into M10 and obtained stable transformants, M10-XRCC4 and M10-CMV, respectively. Figure 2A shows the expression of XRCC4 with Ku86 as control examined by Western blotting. It should be noted that XRCC4 expression in M10-XRCC4 cell is driven by a strong promoter, i.e., CMV promoter. However, XRCC4 protein in M10-XRCC4 was less abundant than that in cultured human cells from various origins, e.g., MOLT-4, Nalm-6, M059K, M059J and H1299 (Fig. 2B and data not shown). Densitometric analysis indicated that the abundance of XRCC4 in M10-XRCC4 cell was 19% of that of human T cell leukemia MOLT-4 (Fig. 2B). M10-XRCC4 exhibited increased radioresistance compared to M10-CMV cells (Fig. 2C), indicating that XRCC4 transgene is functional. We also note that M10-XRCC4 cells grew more rapidly than M10-CMV cells: while the population doubling time of M10-CMV was 15.5 hr, that of M10-XRCC4 was 12.9 hr (Fig. 2D). This is in accordance with

Fig. 4. Detergent-resistant form of XRCC4 is associated with chromatin. The pellet remained after extraction with 0.5% NP-40-containing buffer (P-III) was treated with micrococcal nuclease as indicated. The supernatent recovered after centrifugation (F-V) and XRCC4 was detected by Western blotting using anti-FLAG antibody. Arrows with asterisks indicate the hyperphosphorylated form.

Fig. 5. Mobility-shifted form of XRCC4 is hyperphosphorylated form. (A) The pellet remained after extraction with 0.5% NP-40-containing buffer (P-III) was treated with lambda protein phosphatase as indicated. The reaction was stopped by the addition of equal volume of 2xSDS-PAGE loading buffer and heated in boiling water for 5 min. After centrifugation at 16,000 xg for 5 min, the supernatent was examined by Western blotting using anti-FLAG antibody. (B) F-I, F-II, F-III and F-IV were run on the same gel. Note that different volume, i.e., 0.5, 2, 10 and 10 μl, respectively, of F-I, F-II, F-III and F-IV was loaded. Arrows with asterisks indicate the hyperphosphorylated form.
reduced proliferation capacity of cells from XRCC4- or DNA ligase IV- knockout mice,\textsuperscript{33,34} which may reflect the necessity of XRCC4-DNA ligase IV complex in the clearance of spontaneous DNA damages.

**XRCC4 association with chromatin DNA after X-ray irradiation**

It is expected that non-ionic detergent at some appropriate concentration would solubilize free, \textit{i.e.}, not chromatin-bound, XRCC4 and leave chromatin-bound XRCC4 insoluble. We fractionated M10-CMV and M10-XRCC4 cells, either irradiated or left unirradiated, as outlined in Fig. 1. The amount of XRCC4 in F-IV increased dramatically after irradiation (Fig. 3). We also occasionally observed some increase in F-III fraction. XRCC4 in F-I or F-II showed only marginal decrease, if any, after irradiation. Thus only a small fraction of XRCC4 might have been converted to detergent-resistant form (see below).

To examine whether XRCC4 in F-IV had been bound to chromatin, the pellet P-III was treated with micrococcal nuclease to digest DNA. Although a small amount of XRCC4 was recovered in the supernatent F-V even after mock treatment with buffer alone, the treatment with nuclease greatly increased XRCC4 in F-V (Fig. 4). This observation indicated that XRCC4 found in F-IV represented chromatin DNA-bound form.

XRCC4 in F-I and F-II, representing the major population, might be free, \textit{i.e.}, not chromatin-bound form. Although the nature of XRCC4 in F-III is presently unclear, it might be loosely bound to chromatin or to other structures like nuclear matrix. In this regard, ATM was mainly found in F-III in response to the induction of DSBs in the study by Andegeko et al.\textsuperscript{26}

A part of XRCC4 in F-IV and, to a lesser extent, F-III of irradiated cells exhibited slightly reduced electrophoretic mobility as compared to that of unirradiated cells. To examine the possible involvement of phosphorylation, the pellet P-III was treated with lambda protein phosphatase. The phosphatase treatment diminished the retarded form and converted the whole XRCC4 in F-IV into a form that migrated still faster than the leading edge (Fig. 5A). XRCC4 in F-IV of unirradiated cell was also converted into the faster

![Fig. 6](image-url)

**Fig. 6.** Dose response and time course of XRCC4 residence on chromatin. (A) XRCC4 in F-IV 30 min after exposure to various doses of X-ray. (B, C) XRCC4 in F-IV at various times after exposure to 20 Gy (B) or 2 Gy (C) of X-ray. Right-most lane of each panel (labeled “C”) is F-IV prepared from unirradiated M10-CMV as a negative control. Arrows with asterisks indicate the hyperphosphorylated form. Note that unirradiated control (left-most lane) of (C) appeared more intense than those of (A) and (B) due to different exposure and development conditions. (D) Quantification of XRCC4 in F-IV after 2 Gy irradiation. Results from 3 repeated experiments are shown with different symbols and the mean is indicated with solid line. Inset shows the magnification of time range within 1 hr.
migrating form. We also note that, when F-I, F-II, F-III and F-IV were resolved on the same gel, the position of F-I and F-II corresponded to that of the leading edge of XRCC4 in F-IV of irradiated cells (Fig. 5B). These results collectively indicated that the retarded form of XRCC4 seen in F-IV of irradiated cells was the hyperphosphorylated form and also that XRCC4 protein had undergone constitutive phosphorylation.

The increase of XRCC4 in F-IV was roughly proportional to radiation dose (Fig. 6A). It was evident as early as 0.1 hr after 20 Gy irradiation. It reached the maximum at 0.5 to 1 hr after irradiation and was seen up to 4 hr albeit at a reduced level (Fig. 6B). In the case of 2 Gy irradiation, however, the amount of XRCC4 in F-IV was maximal immediately after irradiation and it decreased rapidly to nearly basal level within 0.5 hr after irradiation (Fig. 6C, D). Thus, the association of XRCC4 with chromatin is an early and sensitive response to ionizing radiation.

Quantification of chromatin-associated XRCC4

We assessed the proportion of XRCC4 in F-IV by resolving F-I to F-IV on the same gel followed by quantitative

| Table 1. Quantification of XRCC4 distribution in each fraction. |
|---------------------------------------------------------------|
|                | F-I        | F-II       | F-III      | F-IV      |
| Experiment 1   |            |            |            |            |
| Uniradiated    | SI%        | SI%        | SI%        | SI%        |
| 20 Gy, 30 min  | 969,451    | 95.9       | 131,494    | 3.3        |
|                | 159,510    | 0.79       | 29,776     | 0.07       |
| Experiment 2   |            |            |            |            |
| Uniradiated    | SI%        | SI%        | SI%        | SI%        |
| 20 Gy, 30 min  | 823,609    | 92.0       | 220,734    | 6.2        |
|                | 95,412     | 0.53       | 465,926    | 1.30       |
| Experiment 3   |            |            |            |            |
| Uniradiated    | SI%        | SI%        | SI%        | SI%        |
| 20 Gy, 30 min  | 246,780    | 90.6       | 68,071     | 6.2        |
|                | 122,266    | 2.24       | 99,396     | 0.91       |

SI, signal intensity. Note that F-I, F-II and F-III had been diluted 40-, 10- and 2-fold, respectively, before loading onto SDS-PAGE.

We assessed the proportion of XRCC4 in F-IV by resolving F-I to F-IV on the same gel followed by quantitative

Fig. 7. Quantification of chromatin bound XRCC4. F-IV from four experiments was resolved on SDS-PAGE along with purified XRCC4/DNA ligase IV (Trevigen Inc.) followed by Western blotting analysis using rabbit polyclonal antibody against XRCC4. The concentration of XRCC4 in purified fraction had been determined by silver staining after SDS-PAGE using BSA as the standard. The molar amount was calculated assuming that the molecular mass of XRCC4 is 38 kDa. Numbers below the photograph are the signal intensity of XRCC4.
Western blotting (Fig. 5B). From three independent experiments, we calculated that approximately 1% of total XRCC4 resided in F-IV 30 min after 20 Gy irradiation (Table 1).

Given the quasi-linear relationship between X-ray dose and the increase of chromatin-bound XRCC4, we sought to assess the stoichiometry between XRCC4 and double-strand break. To obtain the number of XRCC4 molecules, F-IV from four experiments was re-run simultaneously with purified XRCC4 (Fig. 7), the concentration of which had been obtained by silver staining analysis of SDS-PAGE gel using bovine serum albumin as the standard. It was calculated that the number of XRCC4 molecules in F-IV was $4.6 \times 10^3$ per cell on average, ranging from $2.0 \times 10^3$ to $7.8 \times 10^3$. Supposing 1 Gy X-ray irradiation would produce ~40 DSBs,\(^{35}\) i.e., ~80 double-stranded DNA ends, XRCC4 molecules might bind to one DSB. As it is generally thought that one XRCC4-DNA ligase IV complex consists of two XRCC4 molecules and one DNA ligase IV molecule,\(^{36-38}\) only one or few XRCC4-DNA ligase IV complex might be recruited to each DNA end.

**Role of DNA-PKcs in chromatin recruitment of XRCC4**

As described above, we observed a hyperphosphorylated form in chromatin-bound XRCC4. Our earlier study demonstrated that ionizing radiation induced DNA-PK-mediated phosphorylation of XRCC4 accompanying reduction in electrophoretic mobility.\(^{32}\) Therefore, we were interested whether phosphorylation by DNA-PK is required for the radiation-induced chromatin binding of XRCC4. First, we examined the effect of wortmannin, an inhibitor of DNA-PKcs as well as other structurally related kinases. XRCC4 in F-IV was not reduced by the treatment with wortmannin, although the hyperphosphorylated form was diminished (Fig. 8A, Table 2).

We next knocked down DNA-PKcs expression by siRNA. For this purpose, we constructed three vectors expressing siRNA for different regions of DNA-PKcs and transfected them to M10-XRCC4 cells. We took several stable clones for each construct and found that one construct could suppress DNA-PKcs protein expression almost completely. Like XRCC4, DNA-PKcs in F-IV increased after irradiation in control GFP siRNA-expressing cells, but not in DNA-PKcs

**Fig. 8.** Effects of inhibitors and siRNA of DNA-PKcs on radiation-induced chromatin binding of XRCC4. (A) Wortmannin was added 30 min prior to 20 Gy X-ray or mock irradiation at the final concentration of 10 μM. (B) M10-XRCC4 was transfected with siRNA expressing vector directed to DNA-PKcs or GFP, as a control, and stable transformants were examined. Cells were harvested 30 min after 20 Gy X-ray or mock irradiation and examined as described in Fig. 3. Note that, in both A and B, different volume, i.e., 0.5, 2, 10 and 10 μl, respectively, of F-I, F-II, F-III and F-IV was loaded for the analyses of XRCC4. In B, 5 μl of each fraction was examined for DNA-PKcs. Five μl of F-IV is examined for PCNA as the loading control. Arrows with asterisks indicate the hyperphosphorylated form.
siRNA-expressing cells (Fig. 8B). Radiation-induced increase of XRCC4 in F-IV was still observed in DNA-PKcs siRNA-expressing cells, although reduction to 65% or 31% was observed in some experiments (Fig. 8B and Table 2). There was a reduction of hyperphosphorylated form in DNA-PKcs siRNA-expressing cells. Although we cannot presently exclude the possibility that residual DNA-PKcs, albeit hardly detectable, recruited XRCC4 to damaged chromatin, the extent of reduction in the chromatin fraction was much greater for DNA-PKcs compared to XRCC4. These results in the aggregate indicated that the phosphorylation of XRCC4 by DNA-PK and even the presence of DNA-PKcs were not required for the radiation-induced chromatin-recruitment of XRCC4.

### DISCUSSION

It has been difficult to capture the movement of NHEJ enzymes to DSB sites, especially relying on immuno-fluorescence techniques. The present study using sequential extraction with detergent-containing buffer demonstrated the binding of XRCC4 to chromatin DNA after irradiation. Through quantitative analyses, it was estimated that only one or few XRCC4 molecules might be recruited to each DNA end. In addition, the residence of XRCC4 on chromatin might be very transient, particularly after the irradiation with small doses. The present observation might explain reasonably why it has been difficult to capture the movement of NHEJ enzymes to DSB sites.

Drouet et al. reported the movement of NHEJ molecules in response to DSB induction by neocarzinostatin or bleomycin using similar approach. However, there are several differences between the results of two studies. First, they observed that DNase I treatment released DNA-PKcs and Ku but not XRCC4 and DNA ligase IV, leading to the idea that XRCC4 and DNA ligase IV were bound to nuclear matrix or other structure rather than chromatin itself. In the present study, XRCC4 retained after buffer extraction could be released by micrococcal nuclease treatment, indicating its binding to chromatin DNA. Second, in their study, although the movement of XRCC4 was not affected by wortmannin, it was diminished in M059J Fus1 cells, lacking DNA-PKcs. This led them to the conclusion that DNA-PKcs is necessary for the movement, although the phosphorylation is unnecessary. Our results agreed with their results on the dispensability of phosphorylation. However, in the present study, siRNA-mediated knocking-down of DNA-PKcs did not diminish the chromatin binding of XRCC4, indicating that even the presence of DNA-PKcs was unnecessary. Finally, they mentioned that the movement of NHEJ molecules could be observed only after high doses of irradiation in their study. The present study has demonstrated small but significant increase in the chromatin binding of XRCC4 even after conventional dose, i.e., 2 Gy, of irradiation. These discrepancies may reflect (i) the physiological differences between mouse and human cells, (ii) qualitative difference between drug-induced and radiation-induced DNA damages and/or (iii) technical differences between two studies, as the procedures of cell fractionation are substantially different.

Recent studies using laser irradiation demonstrated XRCC4 accumulation in irradiated area, which did not require DNA-PKcs. When DNA-PKcs is not involved, by what mechanism is XRCC4 recruited to damaged chromatin DNA? Ku might be mediating the interaction between XRCC4 and DSB via DNA ligase IV. Another possibility is that XRCC4 moves to a DSB site autonomously due to its intrinsic DNA end-binding activity. Additionally, recent studies by others have suggested that NHEJ is more sophisticated than thought initially and involves many proteins other than DNA-PKcs, Ku, XRCC4-DNA ligase IV, XLF/Cernunnos. Riballo et al. showed that ATM and Artemis, together with Nbs1, Mre11 and 53BP1, function in a sub-pathway of NHEJ that repairs approximately 10% of DSBs, probably those require DNA end processing. Another study by Iwabuchi et al. suggested three parallel, but mutually crosstalk, pathways of NHEJ, i.e., core pathway mediated by DNA-PKcs and Ku, ATM-Artemis pathway and 53BP1 pathway, all of which finally converge on XRCC4-DNA ligase IV. Furthermore, XRCC4 was shown to interact with polynucleotide kinase (PNK) or aprataxin (APTX), depending on the phosphorylation by casein kinase II. These proteins may be required for the repair of subset, at least, of DNA-PKcs siRNA DNA-PKcs – – + +
IR – + – +

| Experiment 1b | 2,391,181 | 6,019,566 | 2,431,534 | 4,773,683 | 0.65 |
| Experiment 2c | 2,029,428 | 3,847,426 | 1,401,475 | 1,962,936 | 0.31 |
| Experiment 3d | 789,728 | 3,220,099 | 1,116,470 | 2,363,692 | 0.51 |

The values are signal intensity expressed in arbitrary unit.

| Wortmannin | 1 | 2 | 3 | 4 | Ratiob |
| Experiment 1b | 1,292,881 | 4,900,020 | 890,462 | 6,124,111 | 1.76 |
| Experiment 2c | 399 | 19,801 | 3,433 | 100,687 | 5.01 |

**Table 2.** Quantitative analysis of the effects of wortmannin and DNA-PKcs siRNA.

|  | 1 | 2 | 3 | 4 | Ratioa |
| --- | --- | --- | --- | --- | --- |
| Wortmannin | – | – | + | + | |
| IR | – | + | – | + | |
| Experiment 1b | 2,391,181 | 6,019,566 | 2,431,534 | 4,773,683 | 0.65 |
| Experiment 2c | 22,706 | 68,056 | 8,588 | 120,929 | 2.47 |
| Experiment 3d | 2,029,428 | 3,847,426 | 1,401,475 | 1,962,936 | 0.31 |

Obtained by capturing chemiluminescence directly.

bObtained by the densitometry of film.

aChange in the radiation-induced chromatin-association of XRCC4 by DNA-PK and even the presence of DNA-PKcs was unnecessary.
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