

Ionizing radiation-induced XRCC4 phosphorylation is mediated through ATM in addition to DNA-PK

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Abstract: XRCC4 (X-ray cross-complementation group 4) is a protein associated with DNA ligase IV, which is thought to join two DNA ends at the final step of DNA double-strand break repair through non-homologous end-joining. It has been shown that, in response to irradiation or treatment with DNA damaging agents, XRCC4 undergoes phosphorylation, requiring DNA-PK. Here we explored possible role of ATM, which is structurally related to DNA-PK, in the regulation of XRCC4. The radiosensitizing effects of DNA-PK inhibitor and/or ATM inhibitor were dependent on XRCC4. DNA-PK inhibitor and ATM inhibitor did not affect the ionizing radiation-induced chromatin recruitment of XRCC4. Ionizing radiation-induced phosphorylation of XRCC4 in the chromatin-bound fraction was largely inhibited by DNA-PK inhibitor but further diminished by the combination with ATM inhibitor. The present results indicated that XRCC4 phosphorylation is mediated through ATM as well as DNA-PK, although DNA-PK plays the major role. We would propose a possible model that DNA-PK and ATM acts in parallel upstream of XRCC4, regulating through phosphorylation.

Keywords: DNA double-strand break repair, non-homologous end-joining, XRCC4, DNA-PKcs, ATM

Introduction

DNA double-strand break (DSB) is considered the most critical type of DNA damage, inducible by ionizing radiation (IR) or a subset of drugs used in

cancer chemotherapy. Eukaryotic cells repair DSBs mainly through two mechanisms, *i.e.*, homologous recombination and non-homologous end-joining (NHEJ).¹⁾ In NHEJ in vertebrate cells, seven molecules playing pivotal role have been identified: Ku70, Ku86 (also known as Ku80), DNA-PKcs (DNA-dependent protein kinase catalytic subunit), Artemis, XRCC4, DNA ligase IV (Lig4) and XRCC4-like factor (XLF, also known as Cernunnos). DNA-PKcs and Ku, the heterodimer of Ku70 and Ku86, consists a protein kinase complex, termed DNA-PK, which binds to and is activated by DSB.^{1);2)} Artemis is an endo- and exo-nuclease for the processing of hairpin or overhang structure. XRCC4 is constitutively associated with Lig4, which joins two DSB ends, conferring the stability and stimulates the ligation and adenylation activities.³⁾⁻⁷⁾ XLF is shown to stimulate the Lig4 activity toward incompatible or mismatched DNA ends.¹⁾

There are lines of evidence indicating the requirement for the kinase activity of DNA-PKcs in its NHEJ function.¹⁾ Kinase-dead mutant of DNA-PKcs (K3752M or K3752R) can restore at most

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Abbreviations: XRCC4: X-ray cross-complementation group 4; ATM: ataxia telangiectasia mutated; DSB: double-strand break; IR: ionizing radiation; NHEJ: non-homologous end-joining; DNA-PKcs: DNA-PK catalytic subunit; Lig4: DNA ligase IV; XLF: XRCC4-like factor; PIKK: phosphatidylinositol 3-kinase-related kinase; CBS: calf bovine serum; DMSO: dimethylsulfoxide.

partial NHEJ activity in DNA-PKcs-deficient cells.^{8),9)} However, it is presently unclear what is/are the *in vivo* phosphorylation target(s) essential for DNA repair,¹⁾ although several studies have shed light on the importance of autophosphorylation of DNA-PKcs itself.¹⁾

Several studies have shown that DNA-PK phosphorylates XRCC4 *in vitro*, decreasing its interaction with DNA, although the significance of these phenomena is presently unclear.^{4),10),11)} Moreover, it has been also shown that XRCC4 undergoes phosphorylation in living cells in response to radiation or treatment with DSB-inducing agent in a manner dependent on DNA-PKcs.^{12)–15)} Several groups, employing mass spectrometry, identified Ser260 and Ser318 as the major phosphorylation sites in XRCC4 by DNA-PK *in vitro*.^{16)–19)} However, it is presently unclear whether these sites are phosphorylated in living cells, especially, in response to DNA damage. Furthermore, the XRCC4 mutants lacking these phosphorylation sites could fully restore radioresistance and V(D)J recombination in XRCC4-deficient XR-1 cells, which is derived from CHO (Chinese hamster ovary) cell, and also exhibited normal activity in DNA joining reaction in cell-free system, leading to the conclusion that XRCC4 phosphorylation by DNA-PK was unnecessary for these functions.^{17),18)} Nevertheless, these results did not exclude the possible existence of additional phosphorylation site(s) with functional importance. Thus further studies are necessary to clarify the biological significance of XRCC4 phosphorylation in DSB repair through NHEJ.

DNA-PKcs is a member of the phosphatidylinositol 3-kinase-related kinase (PIKK) family.²⁰⁾ Another member of PIKK family is ATM, which is mutated in a genetic disorder, ataxia telangiectasia.²¹⁾ ATM binds to and is activated by DSB via NBS1/MRE11/RAD50 (Nijmegen breakage syndrome 1, meiotic recombination 11, radiation sensitivity 50, respectively) complex.²²⁾ DNA-PK and ATM have also similarities in some biochemical properties, *e.g.*, preferring serine-glutamine (SQ) or threonine-glutamine (TQ) sequences as the phosphorylation targets.^{23)–26)} Thus, some part, at least, of the functions of DNA-PK and ATM seems to be interchangeable. In response to ionizing radiation, histone H2AX undergoes phosphorylation at Ser139 (the phosphorylated form is termed γ -H2AX), which is thought to recruit DNA repair and DNA damage response proteins, *e.g.*, MDC1. This phosphorylation is shown to be mediated through ATM and DNA-

PK.^{27),28)} Nevertheless, it is obvious that they have distinct functions; loss of DNA-PKcs manifests defective NHEJ, whereas loss of ATM manifests defective cell cycle checkpoint. It would be also noted that the combined mutation in *Prkdc* (the gene encoding DNA-PKcs) and *Atm* genes in mouse results in synthetic lethality at an early stage of embryogenesis,^{29),30)} indicating a complementary function of DNA-PK and ATM therein.

In our earlier study, radiation-induced phosphorylation of XRCC4 was diminished in DNA-PKcs-deficient human glioma M059J cells and also suppressed by the treatment with wortmannin, which inhibits DNA-PK activity.¹²⁾ On the other hand, XRCC4 phosphorylation remained in fibroblast derived from ataxia-telangiectasia patients, albeit somewhat reduced. These observations led us to the conclusion that DNA-PKcs, but not ATM, is required for the radiation-induced phosphorylation of XRCC4. However, it should be noted that wortmannin acts not only on DNA-PK but also on ATM. In addition, it is reported that M059J has a frameshift mutation in *ATM* gene, resulting in the truncation of the product.³¹⁾ Considering these, our earlier study¹²⁾ had not entirely excluded the possible contribution of ATM in XRCC4 phosphorylation.

These considerations prompted us to revisit the role of ATM in the radiation-induced phosphorylation of XRCC4.

Material and methods

Cell culture, chemicals and irradiation. Murine leukemia L5178Y-derived, XRCC4-deficient cell line M10^{32),33)} was originally obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) with the permission of Dr. Koki Sato (Kinki University). The *Xrcc4* gene in M10 harbors a mutation A370T, resulting in the change of Arg124 to stop codon.³³⁾ Although one of the alleles is not mutated, *Xrcc4* is expressed exclusively from the mutated allele.³³⁾ The full-length, wild-type human *XRCC4* cDNA was obtained by polymerase chain reaction (PCR) from the cDNA pool of human T cell leukemia MOLT-4 cells and inserted into p3xFLAG-CMV10 vector using *EcoRI* and *BglII*.³⁴⁾ The nucleotide sequence of *XRCC4* cDNA obtained above was identical to XM.005248595 and NM.022406, which are registered in National Center for Biotechnology Information (NCBI). M10-XRCC4 and M10-CMV cells were obtained by the introduction of human *XRCC4* cDNA expressing vector and the empty vector as a control, respectively.³⁴⁾ The cells were cultured in

RPMI1640 medium supplemented with 10% calf bovine serum (CBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 10 µM 2-mercaptoethanol at 37 °C in humidified atmosphere containing 5% CO₂. CBS was purchased from HyClone (Logan, UT, USA) and heated at 56 °C for 30 min prior to use. Other agents were purchased from Nacalai Tesque (Kyoto, Japan).

DNA-PK inhibitor, NU7441,³⁵⁾ and ATM inhibitor, KU55933,³⁶⁾ were purchased from Tocris Bioscience (Bristol, UK) and EMD Biochemicals (San Diego, CA, USA), respectively, and dissolved in dimethylsulfoxide (DMSO) at the concentration of 5 mM. Wortmannin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO at the concentration of 10 mM. All of these chemicals were kept at -20 °C and added to the culture medium at the final concentration of 10 µM 1 hr prior to irradiation.

The cells were irradiated using ⁶⁰Co γ-ray source (222 TBq as of February 2010).³⁷⁾ The cells were placed at the distance of 90 cm from the center of the source. The dose rate was approximately 0.8 Gy/min. The cellular radiosensitivity was assessed in terms of their colony forming ability in soft agarose. Appropriate number of cells were suspended in 4 ml of RPMI1640 medium supplemented with 15% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 µM 2-mercaptoethanol and 0.2% agarose and plated onto a 60 mm-plastic dish. After culturing for 10 to 12 days, visible colonies were counted. Plating efficiency was calculated as the number of colony divided by the number of plated cells. Surviving fraction was calculated as the plating efficiency of irradiated cells divided the plating efficiency of unirradiated control.

Preparation of chromatin-bound fraction and analysis of XRCC4 phosphorylation. Chromatin bound-fraction, in which phosphorylated XRCC4 is enriched, was prepared by sequential extraction with increasing concentration of Nonidet P-40 as we described earlier.^{34),37)} Typically, 10⁷ cells were 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 g for 5 min. The supernatant was recovered as F-I, containing free XRCC4, not bound to chromatin. The precipitate was resuspended in 150 µl of the same buffer and immediately centrifuged

at 1,000 g for 5 min. The precipitate 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 centrifuged at 16,000 g for 5 min and the precipitate was suspended in 150 µl of 2 X sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer (125 mM Tris-HCl (pH 6.8), 4% w/v sodium lauryl sulfate, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.02% w/v bromophenolblue, 0.01% crystalviolet) and heated in boiling water for 5 min. After centrifugation at 16,000 g for 5 min, the supernatant was recovered as F-IV, which was shown to represent the chromatin-bound fraction.³⁴⁾

The phosphorylation status of XRCC4 was examined by Western blotting analysis as we described earlier.^{34),37)} For better separation of the hyperphosphorylated form from hypophosphorylated form, 8% polyacrylamide gel, containing 20 µM Phos-tagTM acrylamide (Wako Pure Chemical, Wako, Saitama, Japan) and 80 µM MnCl₂, was used as the separating gel. Anti-FLAG mouse monoclonal antibody (clone M2), labeled with horseradish peroxidase (Sigma-Aldrich), was used to detect XRCC4, which was tagged with FLAG.

Results

Radiosensitizing effects of DNA-PK/ATM inhibitors depend on XRCC4. To clarify the functional interrelationship among XRCC4, DNA-PK and ATM, we examined the effects of DNA-PK/ATM inhibitors on the radiosensitivity of M10-XRCC4 and M10-CMV cells.

The radiosensitivity of M10-XRCC4 was increased by DNA-PK inhibitor NU7441 and, albeit to somewhat lesser extent, by ATM inhibitor KU55933 (Fig. 1A). Combination of DNA-PK inhibitor and ATM inhibitor resulted in greater radiosensitization than either inhibitor alone. On the other hand, DNA-PK inhibitor and ATM inhibitor, separately or in combination, did not affect the radiosensitivity of M10-CMV to a discernable extent (Fig. 1B). These results collectively indicated that the radiosensitizing effect of DNA-PK inhibitor and that of ATM inhibitor requires XRCC4, implying that DNA-PK and ATM acts upstream of XRCC4 in parallel.

We also examined the effects of wortmannin, which inhibits DNA-PK and ATM as well as phosphatidylinositol 3-kinase. Wortmannin increased the radiosensitivity of M10-CMV as well as M10-XRCC4 (Fig. 1C, D). In conjunction with the above results using specific inhibitors toward DNA-PK and ATM, these results indicated that other wortmannin-

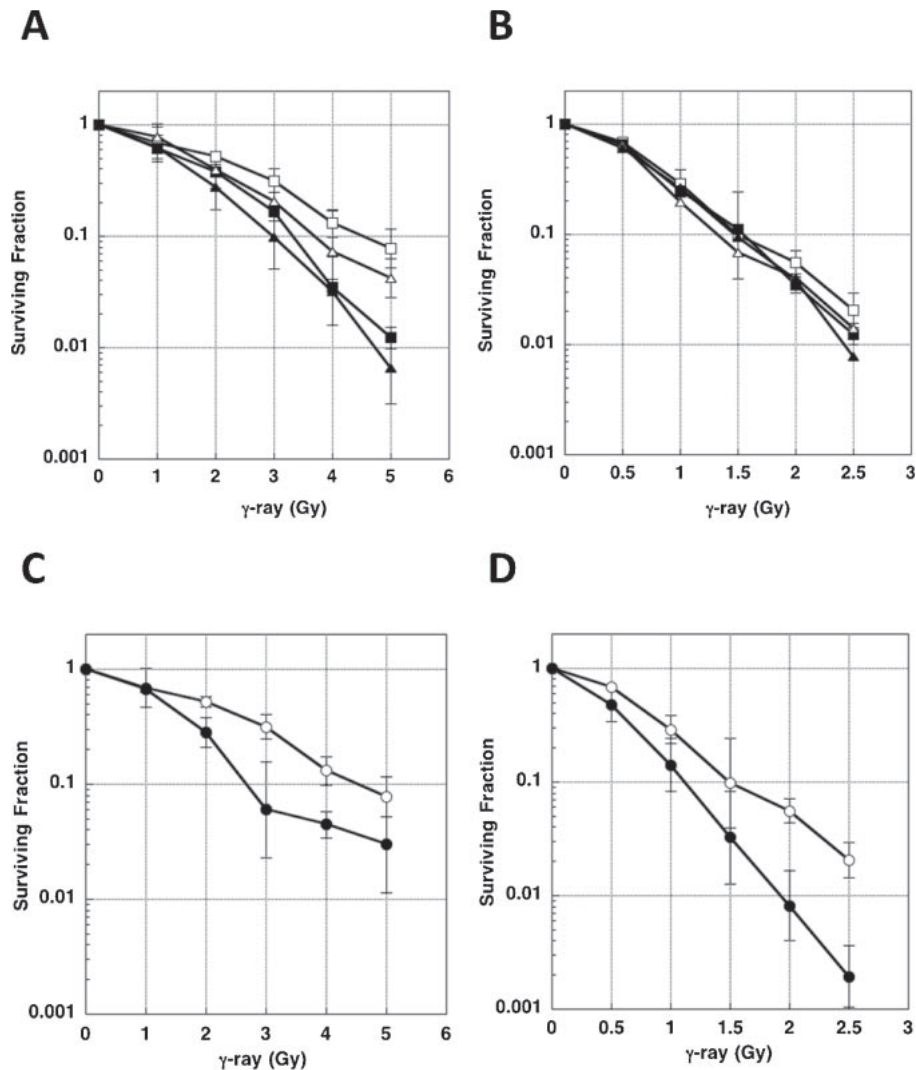


Fig. 1. Radiosensitizing effects of DNA-PK/ATM inhibitors on cells with and without functional XRCC4. (A, B) M10-XRCC4 (A) and M10-CMV (B) cells were treated with DMSO, as vehicle control, (open squares), 10 μ M of DNA-PK inhibitor NU7441 alone (filled squares), 10 μ M of ATM inhibitor KU55933 alone (open triangles) or 10 μ M of NU7441 and KU55933 in combination (filled triangles). (C, D) M10-XRCC4 (C) and M10-CMV (D) cells were treated with DMSO, as vehicle control, (open circles) or 10 μ M of wortmannin (closed circles). The average of results from more than three experiments is shown, with the exception of M10-CMV treated with KU55933 alone or NU7441 and KU55933 in combination in B, which were derived from a single experiment. The error bars represent the standard deviations.

sensitive kinase might contribute to the radiosensitizing effect.

ATM in addition to DNA-PK contributes to radiation-induced phosphorylation of XRCC4. In our earlier study, we demonstrated that the chromatin binding of XRCC4, as detected by the resistance to extraction using a detergent Nonidet P-40, increased after irradiation.³⁴ We also noted that a part of XRCC4 in the chromatin-bound fraction from irradiated cells exhibited a slightly reduced electrophoretic mobility because of phosphorylation.

As it was also found that XRCC4 had undergone constitutive phosphorylation even without irradiation,³⁴ the form showing reduced electrophoretic mobility in truth represents a hyperphosphorylated form, which we would like to denote " γ -XRCC4" for γ -irradiation-inducible form of XRCC4.

We examined the abundance and phosphorylation status of XRCC4 in F-I, which is considered free, non-chromatin-bound fraction, and that in F-IV, which is considered the chromatin-bound fraction, in M10-XRCC4 cells after irradiation. In our earlier

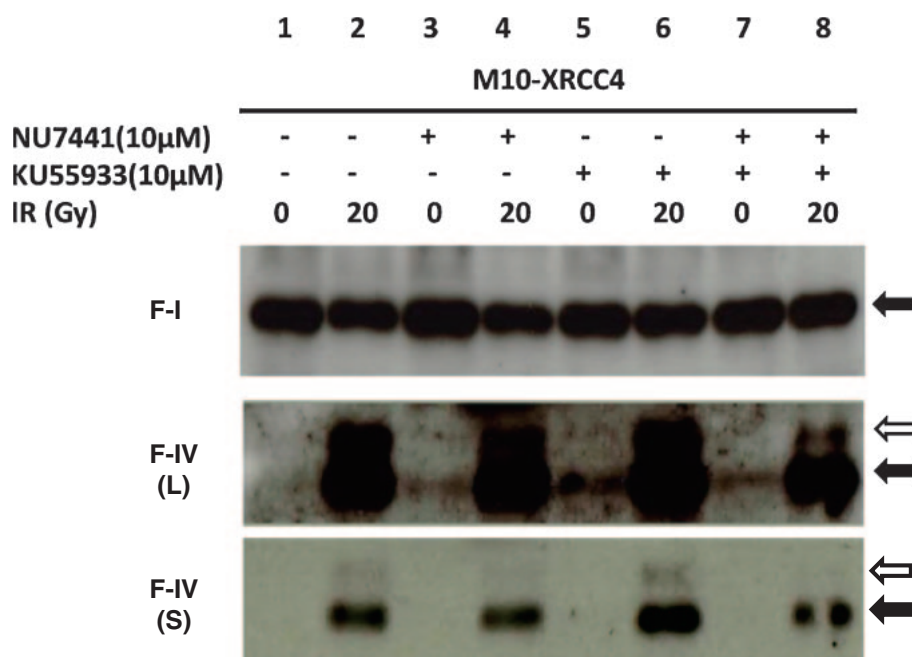


Fig. 2. Ionizing radiation-induced chromatin binding and phosphorylation of XRCC4. M10-XRCC4 cells were harvested 1 hr after γ -ray irradiation (IR) as indicated. F-I represents free, non-chromatin-bound fraction, whereas F-IV represents chromatin-bound fraction. Hypophosphorylated form of XRCC4 is indicated by filled arrows in F-I and F-IV and hyperphosphorylated form is indicated with an open arrow in F-IV.

study,¹²⁾ the hyperphosphorylated form of XRCC4 was seen 0.5–4 hr post-irradiation and appeared maximal 1 hr post-irradiation in human T cell leukemia MOLT-4 cells. In our preliminary experiment, we observed that the bands of hyperphosphorylated form were clearly detected after irradiation with 20 Gy or higher doses and the intensity of the bands increased in a manner roughly proportional to the radiation doses. Therefore, we analyzed XRCC4 1 hr after 20 Gy γ -irradiation. XRCC4 in F-I did not change appreciably 1 hour after irradiation with 20 Gy of γ -ray (Fig. 2, top). On the other hand, XRCC4 in F-IV was increased after 20 Gy γ -irradiation (Fig. 2, bottom, compare lanes 2, 4, 6 and 8 with lanes 1, 3, 5 and 7, respectively), in agreement with our earlier study.³⁴⁾ Neither DNA-PK inhibitor NU7441 nor ATM inhibitor KU55933, separately or in combination, reduced the radiation-induced chromatin binding of XRCC4 significantly (compare the total signals of lanes 4, 6 and 8 with that of lane 2). These results indicated that the kinase activities of DNA-PK and ATM are not required for chromatin recruitment of XRCC4 in response to radiation-induced DNA damage.

The appearance of hyperphosphorylated XRCC4 in chromatin-bound fraction after 20 Gy γ -irradiation

was suppressed significantly, although not completely, by DNA-PK inhibitor NU7441 (Fig. 2, bottom, compare the upper band, pointed by open arrow, in lane 4 with that in lane 2). Although ATM inhibitor KU55933 did not reduce hyperphosphorylated XRCC4 significantly (compare the upper band in lane 6 with that in lane 2), the combination of DNA-PK inhibitor and ATM inhibitor diminished hyperphosphorylated XRCC4 to a greater extent than DNA-PK inhibitor alone (compare the upper band in lane 8 with that in lane 4). These results indicated that not only DNA-PK but also ATM contributes to the production of hyperphosphorylated XRCC4 complementarily, although DNA-PK might play a major role.

Discussion

Here we showed that XRCC4 phosphorylation in response to radiation is mediated through ATM as well as DNA-PK, although DNA-PK plays the major role. In conjunction with the effects of DNA-PK inhibitor and ATM inhibitor on radiosensitivity, we would propose a possible model that DNA-PK and ATM act in parallel upstream of XRCC4, regulating through phosphorylation (Fig. 3). We also showed that the radiation-induced chromatin recruitment of

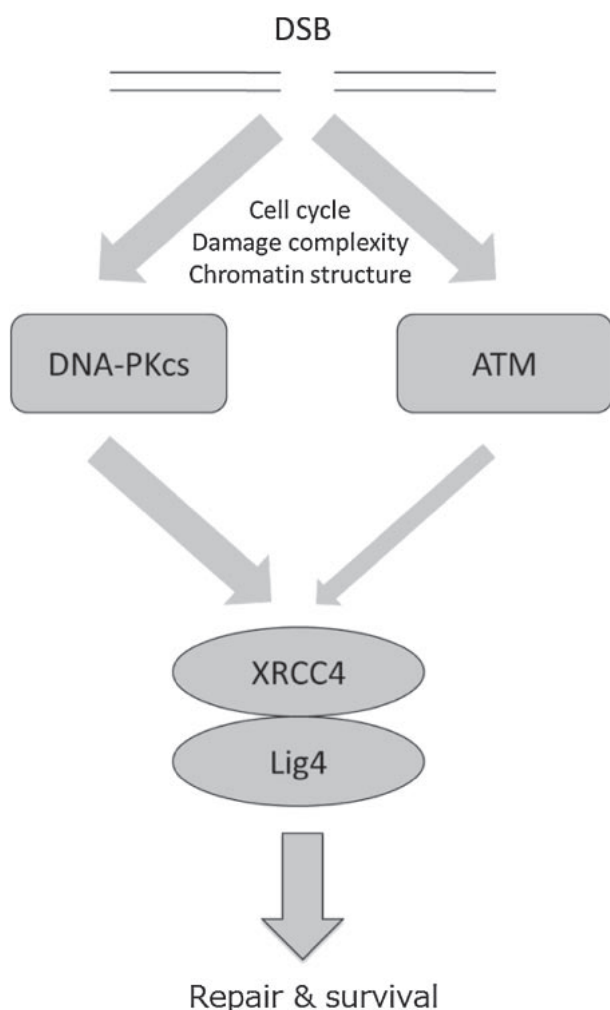


Fig. 3. A possible model for the functional interrelationship among XRCC4, DNA-PK and ATM. DNA-PK and ATM are activated by DSB and phosphorylate XRCC4, leading to repair and cell survival. DNA-PK plays a major role in XRCC4 phosphorylation. Usage of DNA-PK or ATM for XRCC4 phosphorylation may depend on cell cycle stage, complexity of DNA damage or chromatin structure. DNA-PK and ATM may phosphorylate the same or different site(s) in XRCC4 and bring the same or different consequences, which remain to be clarified. Note that F-I and F-IV (S) were exposed for 15 sec, whereas F-IV (L) was exposed for 3 min.

XRCC4 was not dependent on the kinase activities of DNA-PK and ATM. Therefore, XRCC4 phosphorylation by DNA-PK and/or ATM would probably occur following the recruitment of XRCC4 to DSB site. Additionally, wortmannin exhibited a significant radiosensitizing effect on XRCC4-deficient cells, indicating the involvement of wortmannin-sensitive molecule(s) other than DNA-PK and ATM, *e.g.*, phosphatidylinositol 3-kinase in radiosensitization by

wortmannin. Presently, we cannot entirely exclude this possibility that heterologous experiment system employed here, *i.e.*, introducing human *XRCC4* cDNA into murine XRCC4-deficient cells, might have some influence on the results. Indeed, the identity of amino acid sequence of XRCC4 between human and mouse is 74%. However, the phosphorylation sites identified so far are conserved between human and mouse.

It is not presently clear whether the function of DNA-PK and ATM in the phosphorylation of XRCC4 is "overlapping" or "separate" (Fig. 3). Although ATM as well as DNA-PK is implicated in NHEJ, they might have distinct roles, depending on cell cycle stage, complexity of DNA damage and chromatin structure.^{38),39)} Thus, XRCC4 might be phosphorylated by DNA-PK or ATM in a manner dependent on these factors. In addition, it is currently unknown whether the site(s) and the consequence of the phosphorylation by these two kinases are the same or different. Ser260 and Ser320 were identified as the major phosphorylation sites by DNA-PK, but the disruption of these sites did not exhibit change in NHEJ function.^{17),18)} While DNA-PK and ATM preferentially phosphorylate SQ or TQ sequence, there is a unique SQ sequence, *i.e.*, Ser53, in human XRCC4. However, Mizuta *et al.* reported that XRCC4^{S53A} exhibited no defects in terms of radiosensitivity and V(D)J recombination.⁴⁰⁾ We also observed that XRCC4^{S53A} could fully restore normal radiosensitivity to M10 cells (our unpublished results). These collectively indicate the presence of phosphorylation site(s) by DNA-PK and/or ATM other than Ser260, Ser320 and Ser53 in XRCC4, whose disruption affects NHEJ function. Identification of other phosphorylation site(s) by DNA-PK and/or ATM and the analysis of phosphorylation at each site, *e.g.*, using phosphorylation-specific antibody, are demanded.

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References

- 1) Wang, C. and Lees-Miller, S.P. (2013) Detection and repair of ionizing radiation-induced DNA double strand breaks: New developments in nonhomologous end joining. *Int. J. Radiat. Oncol. Biol. Phys.* **86**, 440–449.
- 2) Gottlieb, T.M. and Jackson, S.P. (1993) The DNA-dependent protein kinase: requirements for DNA ends and association with Ku antigen. *Cell* **72**, 131–142.
- 3) Li, Z., Otevrel, T., Gao, Y., Cheng, H., Seed, B., Stamato, T., Taccioli, G. and Alt, F. (1995) The XRCC4 gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination. *Cell* **83**, 1079–1089.
- 4) Critchlow, S.E., Bowater, B.P. and Jackson, S.P. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Curr. Biol.* **7**, 588–598.
- 5) Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T., Mann, M. and Lieber, M. (1997) Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature* **388**, 492–495.
- 6) Grawunder, U., Zimmer, D. and Lieber, M. (1998) DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. *Curr. Biol.* **8**, 873–876.
- 7) Bryans, M., Valenzano, M. and Stamato, T. (1999) Absence of DNA ligase IV protein in XR-1 cells: evidence for stabilization by XRCC4. *Mutat. Res.* **433**, 53–58.
- 8) Kurimasa, A., Kumano, S., Boubnov, N.V., Story, M.D., Tung, C.-S., Peterson, S.R. and Chen, D.C. (1999) Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining. *Mol. Cell. Biol.* **19**, 3877–3884.
- 9) Kienker, L.J., Shin, E.K. and Meek, K. (2000) Both V(D)J recombination and radioresistance require DNA-PK kinase activity, though minimal levels suffice for V(D)J recombination. *Nucleic Acids Res.* **28**, 2752–2761.
- 10) Leber, R., Wise, T.W., Mizuta, R. and Meek, K. (1998) XRCC4 gene product is a target for and interacts with the DNA-dependent protein kinase. *J. Biol. Chem.* **273**, 1794–1801.
- 11) Modesti, M., Hesse, J. and Gellert, M. (1999) DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity. *EMBO J.* **18**, 2008–2018.
- 12) Matsumoto, Y., Suzuki, N., Namba, N., Umeda, N., Ma, X.-J., Morita, A., Tomita, M., Enomoto, A., Serizawa, S., Hirano, K., Sakai, K., Yasuda, H. and Hosoi, Y. (2000) Cleavage and phosphorylation of XRCC4 protein induced by X-irradiation. *FEBS Lett.* **478**, 67–71.
- 13) Drouet, J., Delteil, C., Lefrancois, J., Concannon, P., Salles, B. and Calsou, P. (2005) DNA-dependent protein kinase and XRCC4-DNA ligase IV mobilization in the cell in response to DNA double strand breaks. *J. Biol. Chem.* **280**, 7060–7069.
- 14) Drouet, J., Frit, P., Delteil, C., de Villartay, J.-P., Salles, B. and Calsou, P. (2006) Interplay between Ku, Artemis, and the DNA-dependent protein kinase catalytic subunit at DNA ends. *J. Biol. Chem.* **281**, 27784–27793.
- 15) Wu, P.-Y., Frit, P., Malivert, L., Revy, P., Biard, D., Salles, B. and Calsou, P. (2007) Interplay between Cernunnos-XLF and nonhomologous end-joining proteins at DNA ends in the cell. *J. Biol. Chem.* **282**, 31937–31943.
- 16) Lee, K.-J., Dong, X., Wang, J., Takeda, Y. and Dynan, W.S. (2002) Identification of human autoantibodies to the DNA ligase IV/XRCC4 and mapping of an autoimmune epitope to a potential regulatory region. *J. Immunol.* **169**, 3413–3421.
- 17) Yu, Y., Wang, W., Ding, Q., Ye, R., Chen, D., Merkle, D., Schriemer, D., Meek, K. and Lees-Miller, S.P. (2003) DNA-PK phosphorylation sites in XRCC4 are not required for survival after radiation or for V(D)J recombination. *DNA Repair (Amst.)* **2**, 1239–1252.
- 18) Lee, K.-J., Jovanovic, M., Udayakumar, D., Bladen, C.L. and Dynan, W.S. (2003) Identification of DNA-PKcs phosphorylation sites in XRCC4 and effects of mutation at these sites on DNA end joining in a cell-free system. *DNA Repair (Amst.)* **3**, 267–276.
- 19) Wang, Y.-G., Nnakwe, C., Lane, W.S., Modesti, M. and Frank, K.M. (2004) Phosphorylation and regulation of DNA ligase IV stability by DNA-dependent protein kinase. *J. Biol. Chem.* **279**, 37282–37290.
- 20) Hartley, K., Gell, D., Smith, C., Zhang, H., Divecha, N., Connelly, M., Admon, A., Lees-Miller, S., Anderson, C. and Jackson, S. (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* **82**, 849–856.
- 21) Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S.R., Simmons, A., Clines, G.A., Sarti, A., Jaspers, N.G.J., Taylor, A.M.R., Arlett, C.F., Miki, T., Weissman, S.M., Lovett, M., Collins, F.S. and Shiloh, Y. (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268**, 1749–1753.
- 22) Falck, J., Coates, J. and Jackson, S.P. (2005) Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature* **434**, 605–611.
- 23) Lees-Miller, S., Sakaguchi, K., Ullrich, S., Appella, E. and Anderson, C. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* **12**, 5041–5049.
- 24) Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson,

- C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y. and Ziv, Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**, 1674–1677.
- 25) Canman, C.E., Lim, D.S., Cimprich, K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B. and Siliciano, J.D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* **281**, 1677–1679.
- 26) Kim, S.-T., Lim, D.-S., Canman, C.E. and Kastan, M.B. (1999) Substrate specificities and identification of putative substrates of ATM kinase family members. *J. Biol. Chem.* **274**, 37538–37543.
- 27) Burma, S., Chen, B.P., Murphy, M., Kurimasa, A. and Chen, D.J. (2001) ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J. Biol. Chem.* **45**, 42462–42467.
- 28) Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Löbrich, M. and Jeggo, P.A. (2004) ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res.* **64**, 2390–2396.
- 29) Gurley, K.E. and Kemp, C.J. (2001) Synthetic lethality between mutation in Atm and DNA-PKcs during murine embryogenesis. *Curr. Biol.* **11**, 191–194.
- 30) Sekiguchi, J., Ferguson, D.O., Chen, H.T., Yang, E.M., Earle, J., Frank, K., Whitlow, S., Gu, Y., Xu, Y., Nussenzweig, A. and Alt, F.W. (2001) Genetic interactions between ATM and the non-homologous end-joining factors in genomic stability and development. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3243–3248.
- 31) Tsuchida, R., Yamada, T., Takagi, M., Shimada, A., Ishioka, C., Katsuki, Y., Igarashi, T., Chessa, L., Delia, D., Teraoka, H. and Mizutani, S. (2002) Detection of ATM gene mutation in human glioma cell line M059J by a rapid frameshift/stop codon assay in yeast. *Radiat. Res.* **158**, 195–201.
- 32) Sato, K. and Hieda, N. (1979) Isolation and characterization of mutant mouse lymphoma cell sensitive to methylmethanesulfonate and X-rays. *Radiat. Res.* **78**, 167–171.
- 33) Mori, M., Itsukaichi, H., Nakamura, A. and Sato, K. (2001) Molecular characterization of ionizing-radiation-hypersensitive mutant M10 cells. *Mutat. Res.* **487**, 85–92.
- 34) Kamdar, R.P. and Matsumoto, Y. (2010) Radiation-induced XRCC4 association with chromatin DNA analyzed by biochemical fractionation. *J. Radiat. Res.* **51**, 303–313.
- 35) Leahy, J.J.J., Golding, B.T. and Griffin, R.J. (2004) Identification of a highly potent and selective DNA-dependent protein kinase (DNA-PK) inhibitor (NU7441) by screening of chromenone libraries. *Bioorg. Med. Chem. Lett.* **14**, 6083–6087.
- 36) Hickson, I., Zhao, Y. and Richardoson, C.J. (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Res.* **64**, 9152–9159.
- 37) Liu, S., Liu, X., Kamdar, R.P., Wanotayan, R., Sharma, M.K., Adachi, N. and Matsumoto, Y. (2013) C-terminal region of DNA ligase IV drives XRCC4/DNA ligase IV complex to chromatin. *Biochem. Biophys. Res. Commun.* **439**, 173–178.
- 38) Riballo, E., Kühne, M., Rief, N., Doherty, A., Smith, G.C., Recio, M.J., Reis, C., Dahm, K., Fricke, A., Krempler, A., Parker, A.R., Jackson, S.P., Gennery, A., Jeggo, P.A. and Löbrich, M. (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to γ -H2AX foci. *Mol. Cell* **16**, 715–724.
- 39) Goodarzi, A.A., Noon, A.T., Deckbar, D., Ziv, Y., Shiloh, Y., Löbrich, M. and Jeggo, P.A. (2008) ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol. Cell* **31**, 167–177.
- 40) Mizuta, R., Cheng, H.L., Gao, Y. and Alt, F.W. (1997) Molecular genetic characterization of XRCC4 function. *Int. Immunol.* **9**, 1607–1613.

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