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Asparagine 326 in the extremely C-terminal region of XRCC4 is essential for the cell survival after irradiation

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ABSTRACT

XRCC4 is one of the crucial proteins in the repair of DNA double-strand break (DSB) through non-homologous end-joining (NHEJ). As XRCC4 consists of 336 amino acids, N-terminal 200 amino acids include domains for dimerization and for association with DNA ligase IV and XLF and shown to be essential for XRCC4 function in DSB repair and V(D)J recombination. On the other hand, the role of the remaining C-terminal region of XRCC4 is not well understood. In the present study, we noticed that a stretch of ~20 amino acids located at the extreme C-terminus of XRCC4 is highly conserved among vertebrate species. To explore its possible importance, series of mutants in this region were constructed and assessed for the functionality in terms of ability to rescue radiosensitivity of M10 cells lacking XRCC4. Among 13 mutants, M10 transfectant with N326L mutant (M10-XRCC4^{N326L}) showed elevated radiosensitivity. N326L protein showed defective nuclear localization. N326L sequence matched the consensus sequence of nuclear export signal. Leptomycin B treatment accumulated XRCC4^{N326L} in the nucleus but only partially rescued radiosensitivity of M10-XRCC4^{N326L}. These results collectively indicated that the functional defects of XRCC4^{N326L} might be partially, but not solely, due to its exclusion from nucleus by synthetic nuclear export signal. Further mutation of XRCC4 Asn326 to other amino acids, *i.e.*, alanine, aspartic acid or glutamine did not affect the nuclear localization but still exhibited radiosensitivity. The present results indicated the importance of the extremely C-terminal region of XRCC4 and, especially, Asn326 therein.

1. Introduction

DNA double-strand break (DSB), which is considered the most lethal type of DNA damage, is repaired through homologous recombination (HR) and non-homologous end-joining (NHEJ) in eukaryotic cells. HR attempts to restore the sequence using the homologous chromosome or the sister chromatid as the template, whereas NHEJ attempts to join two ligatable DNA ends directly without the requirement of any template. Therefore, HR is considered more accurate than NHEJ. However, as vertebrate cells can utilize the sister chromatid, but not the homologous chromosome, as the template, HR is available only in the S and G2 phase. On the other hand, NHEJ is considered error-prone but is available throughout the cell cycle. NHEJ is utilized also in V(D)J recombination in immune systems. Defective repair of DSBs can lead to increased radiosensitivity, immunodeficiency, growth retardation and elevated risk of cancer [1].

In NHEJ, a heterodimer of Ku70 and Ku86 first binds to the end of DNA and in turn recruits DNA-PKcs. When the DNA ends are not ready for ligation, they undergo processing by enzymes like Artemis, polynucleotide kinase/phosphatase (PNKP) and DNA polymerases μ/λ. Finally, DNA ends are ligated by DNA ligase IV (LIG4), which is regulated by XRCC4 and XLF [2].

XRCC4 was originally identified as the gene, which can rescue high radiosensitivity and V(D)J recombination defect of Chinese hamster ovary-derived XR-1 cells, which belongs to the complementation group 4 of ionizing radiation-sensitive mutants [3].

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XRCC4 was then shown to be associated with and required for the activity of LIG4 [4,5]. In the cell, XRCC4 stabilizes LIG4 by protecting it from degradation [6]. XLF, also known as Cernunnos, was found as a molecule, which is associated with and shows a structural similarity to XRCC4 [7–9]. XLF is shown to support LIG4 in situations that the ends are not readily ligatable, *e.g.*, in the case of incompatible or mismatched DNA ends [10,11]. It is also suggested that XRCC4 and XLF interact with each other to form a fiber, which might bridge two DNA ends [12].

XRCC4 consists of N-terminal globular head domain (1–115), middle stalk domain (119–203) and C-terminal domain [13–15]. Two XRCC4 molecules form a dimer through coiled-coil interaction mediated through middle stalk domain. The interface with LIG4 also lies in middle stalk domain, especially 173–195. XRCC4 and XLF interact with each other through N-terminal globular head domain. There have been several studies showing that N-terminal ~200 amino acids of XRCC4 are sufficient for its function in DNA repair and V(D)J recombination [16–18].

On the other hand, the function of C-terminal domain has been less identified. C-terminal region is structurally disordered [13–15] and less conserved, *e.g.*, between human and budding yeast, than N-terminal and middle domains. Nevertheless, it contains putative nuclear localization signal and it was shown that the deletion of XRCC4 including this region abrogates its nuclear localization [19]. It has been shown that XRCC4 undergoes phosphorylation at Thr233 by Casein Kinase II, facilitating its interaction with polynucleotide kinase/phosphatase (PNKP) or aprataxin via their forkhead-associated (FHA) domain [20,21]. Moreover, XRCC4 is phosphorylated by DNA-PK *in vitro* [4,16,19] and in response to radiation or treatment with DSB-inducing agent in a manner dependent on DNA-PKcs [22–25]. Mass spectrometric analysis identified Ser260 and Ser318 (note that the latter corresponds to Ser320 in this study due to alternative splicing), although the significance of the phosphorylation remains to be clarified [26–28]. Proteomic analysis revealed XRCC4 phosphorylation at Ser325 and/or Ser326 (corresponding to Ser327 and Ser328 in this study, respectively) in mitosis and in T cell receptor signaling [29,30]. Very recently, Ser326 (corresponds to Ser328 in this study) was shown to be phosphorylated by cyclin-dependent kinase to suppress NHEJ in mitosis, which may lead to genetic instability [31].

In this study, we noticed that a stretch of ~20 amino acids located at the C-terminus of XRCC4 is highly conserved among vertebrate species. To explore its possible importance in DSB repair, we generated and analyzed a series of mutants of amino acids in this region.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Human XRCC4 cDNA had been obtained by polymerase chain reaction (PCR) from the cDNA pool of human T-cell leukemia MOLT-4 cells and integrated into p3XFLAG-CMV-10 vector (Sigma–Aldrich; St. Louis, MO, USA) [32]. To express XRCC4 as a fusion protein with green fluorescent protein (GFP), XRCC4 cDNA was excised from p3XFLAG-CMV-10 vector using restriction enzymes *Eco*RI and *Bam*HI and inserted into pEGFP-C1 vector (Clontech; Mountain View, CA, USA).

Point mutations were introduced using PrimeSTAR Mutagenesis Kit (Takara Bio; Otsu, Shiga, Japan). Primers for mutagenesis are shown in Supplementary materials (Table S1). Correctness of the sequence of entire XRCC4 open reading frame was verified by sequencing for all the constructs.

2.2. Cell culture

The derivative of L5178Y mouse leukemia cell line, M10 cell, was obtained from RIKEN cell bank (Tsukuba, Ibaraki, Japan; Code RCB0136) with permission of Dr. Koki Sato (National Institute of Radiological Sciences and Kinki University). M10 cells harbor mutation in XRCC4 gene (c.A370T, p.R124X), thus are defective in DSB repair and radiosensitive [33,34]. M10 cells were cultured in RPMI1640 medium supplemented with 10% v/v fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol at 37 °C in humidified atmosphere containing 5% CO_2. Human cervical carcinoma cell line HeLa was cultured in DMEM/Ham's F-12 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. FBS was purchased from Hyclone (Logan, UT, USA). Other agents were purchased from Nacalai Tesque (Kyoto, Japan).

2.3. Transfection

For M10 cells, plasmids were transfected using Neon Transfection system (Invitrogen; Carlsbad, CA, USA). Two days after transfection, cells were plated in RPMI1640 medium supplemented with 15% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μM β-mercaptoethanol and 0.2% agarose (Difco). Two weeks after plating, visible colonies were picked up and cultured in G418 containing complete medium for drug selection. After several passages, the stable transformants of different mutated XRCC4 constructs were established.

For HeLa cells, plasmids were transfected using Lipofectamine 2000 Reagent (Invitrogen). To knockdown the endogenous XRCC4 in HeLa cells, small interfering RNA (siRNA) targeting 3′-untranslated region (UTR) was transfected 24 h prior to the transfection of the plasmid. The sequences of RNA duplex were 5′- CUA UGU UUU CUA UUC AUU UdCdT -3′ and 5′- AAA UGA AUA GAA AAC AUA GdTdC -3′, where “d” indicates deoxyribonucleotide. Oligonucleotides were synthesized by Japan Bioservice (Saitama, Japan).

2.4. Fluorescence microscopy

Fluorescence of EGFP was observed with an inverted fluorescence microscope, IX71 (Olympus; Tokyo, Japan). The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI).

2.5. Irradiation

Cells were irradiated using ^{60}Co γ-ray source (222 TBq as of February 2010). The dose rate was measured using ionizing chamber-type exposure dosimeter C-110 (Oyo Giken; Tokyo, Japan).

2.6. Colony formation assay

The radiosensitivity of M10 cells and derivatives was assessed in terms of colony forming ability in soft agarose. Cells were suspended in 1 ml of complete medium when irradiation was performed. After irradiation, cells were diluted and plated in three 6 cm-dish containing 4 ml of plating medium (complete medium containing 0.25% agarose) per sample. After two weeks, colonies containing more than 50 cells were counted. Plating efficiency (P.E.) was calculated as the number of colony divided by the number of plated cells. Surviving fraction (S.F.) was calculated as P.E. of irradiated cells divided by P.E. of unirradiated control. All experiments were repeated at least three times. The average value and standard deviation were plotted in the figures.

2.7. Antibodies and Western blotting

Anti-FLAG monoclonal antibody M2, which had been conjugated with horseradish peroxidase (A8592; Sigma–Aldrich), and anti-XRCC4 rabbit polyclonal antibody generated in our laboratory were used as primary antibodies [32]. For the latter, anti-rabbit immunoglobulin swine antibody, which had been conjugated with horseradish peroxidase (P0447; DAKO; Glostrup, Denmark), was used as secondary antibody. Procedures of Western blotting followed our earlier publications with minor modifications [32,35].

2.8. Treatment with Leptomycin B

Leptomycin B was dissolved in 99.5% ethanol at the concentration of 1 or 50 mg/ml for stock and kept at −20 °C. Leptomycin B was added to the culture medium at the final concentration of 1–100 ng/ml, as indicated, 2 h prior to irradiation.

3. Results

3.1. High degree of amino acid sequence conservation in extremely C-terminal region of XRCC4 among vertebrate species

The amino acid sequences of XRCC4 proteins of various species were retrieved from NCBI database. It was noticed that the sequence of ~20 amino acids at the extremely C-terminus is highly conserved among wide range of vertebrate species including mammal (*Homo sapiens* and *Mus musculus*), bird (*Gallus gallus*), reptile (*Anolis carolinensis*), amphibian (*Xenopus laevis*) and fish (*Danio rerio*) (Fig. 1A). Especially, the amino acids corresponding to Asn326, Pro329, Leu332 and Phe333 of human XRCC4 are conserved among all the species listed here. The amino acids corresponding to Thr323, Asp331, Asp334 and Ile336 of human XRCC4 are conserved in 5 of 6 species. It is also noteworthy that this region is enriched in acidic amino acids, *i.e.*, aspartic acid and glutamic acid. Interestingly, this motif is not found in organisms other than vertebrate. We would denote this region as XECT (XRCC4 extremely C-terminal) region.

3.2. Functional deficiency of N326L mutant of XRCC4

To explore possible importance of XECT region, we created a series of mutants in this region. Mutated XRCC4 cDNA with N-terminal EGFP tag was introduced into XRCC4-deficient M10 cells and stable transformant clones were established. The functionality of mutated XRCC4 was examined in terms of the clonogenic survival of these cells after 2 Gy γ-irradiation (Fig. 1B).

The surviving fraction of M10 was 0.10, whereas that of two transformants with wild-type XRCC4 (M10-XRCC4^{WT}) was 0.60 and 0.65, respectively. Eleven mutants showed similar radiosensitivity to M10-XRCC4^{WT} but two mutants, *i.e.*, M10-XRCC4^{R325F} and M10-XRCC4^{N326L}, showed significantly elevated radiosensitivity. This indicated that XRCC4^{R325F} and XRCC4^{N326L} might be functionally deficient.

In Western blotting, the expression level of XRCC4^{R325F} was found substantially reduced as compared to that of XRCC4^{WT}. Therefore, it cannot be excluded that functional deficiency of XRCC4^{R325F} is simply due to low abundance. On the other hand, the expression level of XRCC4^{N326L} was comparable to that of XRCC4^{WT}.

3.3. Defective nuclear localization of N326L mutant of XRCC4

Subcellular localization of GFP-tagged XRCC4 (GFP-XRCC4) was examined by fluorescent microscopy. As M10 is a round cell with small cytoplasm, HeLa cell was used in this experiment. We considered that the endogeneous XRCC4 might dimerize with mutated XRCC4 and escort it into the nucleus. Therefore, endogeneous XRCC4 was knocked down using siRNA directed to 3′-UTR prior to the transfection with GFP-XRCC4 expression vector. Although GFP-XRCC4^{WT} and GFP-XRCC4^{R325F} localized to the nucleus, GFP-XRCC4^{N326L} was excluded from the nucleus (Fig. 2). GFP-XRCC4^{N326L} showed similar localization even without siRNA-mediated knockdown of endogeneous XRCC4 (Fig. S1).

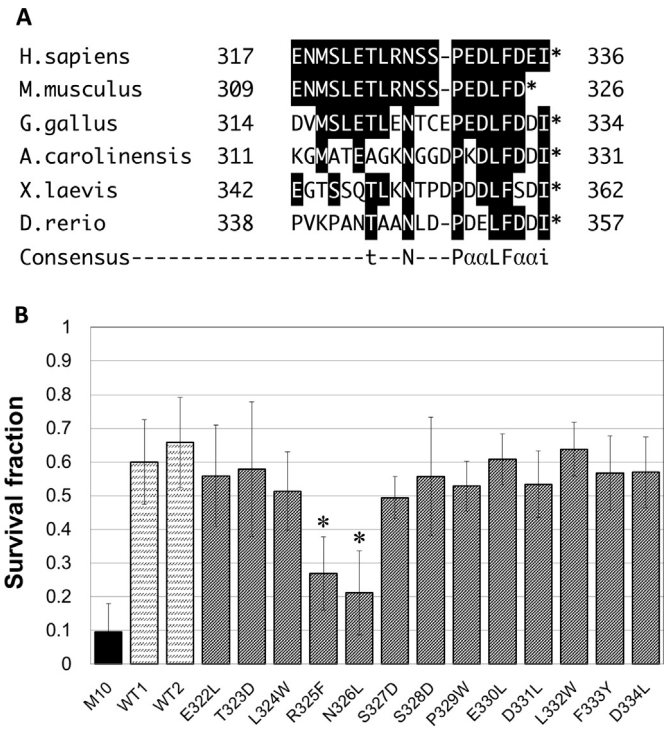

Fig. 1. A: Alignment of the amino acid sequences in the extreme C-termini of XRCC4 proteins from various vertebrate species. The NCBI accession numbers are as follows: *Homo sapiens*, AK_290739.1; *Mus musculus*, XM_001471743.1; *Gallus gallus*, XM_424905.2; *Anolis carolinensis*, XM_003216282; *Xenopus laevis*, NM_001091891.1; *Danio rerio*, NM_200786.1 The amino acids, which are common between human and other species, are shown in white characters. In "Consensus", characters in uppercase show the amino acids, which are common among all of the species and characters in lowercase shows the amino acids, which are common among five out of six species. "α" indicates conserved acidic residues. B: Functionality of mutants of extremely C-terminal region of XRCC4 in terms of clonogenic survival after irradiation. Each mutant was introduced into XRCC4-deficient M10 cells and surviving fraction after 2 Gy of γ-irradiation was obtained by colony formation assay in agarose-containing medium. The mean of results from more than three repeated experiments is shown with the error bar indicating the standard deviation. Asterisk (*) represents statistical significance of surviving fraction in comparison to M10-XRCC4^{WT} ($p < 0.025$).

3.4. Restoration of nuclear localization of N326L by a nuclear export inhibitor, Leptomycin B

Failure of N326L in the nuclear localization was initially unexpected because N326 is not within the nuclear localization signal (NLS). Nuclear export was considered as another possibility. Strikingly, N326L, but not WT, matched the consensus sequence of leucine-rich nuclear export signal (LR-NES) [36–38], which is recognized by exportin1, CRM1 [39] (Fig. 2B). We hypothesized that N326L mutation might have created a synthetic nuclear export signal.

To test this possibility, we examined the effects of an inhibitor of CRM1/exportin1, Leptomycin B [39], on the subcellular localization of N326L. As expected, the treatment with Leptomycin B accumulated GFP-tagged XRCC4 N326L protein in the nucleus (Fig. 2C). This result indicated that failure of N326L in the nuclear localization was due to nuclear export.

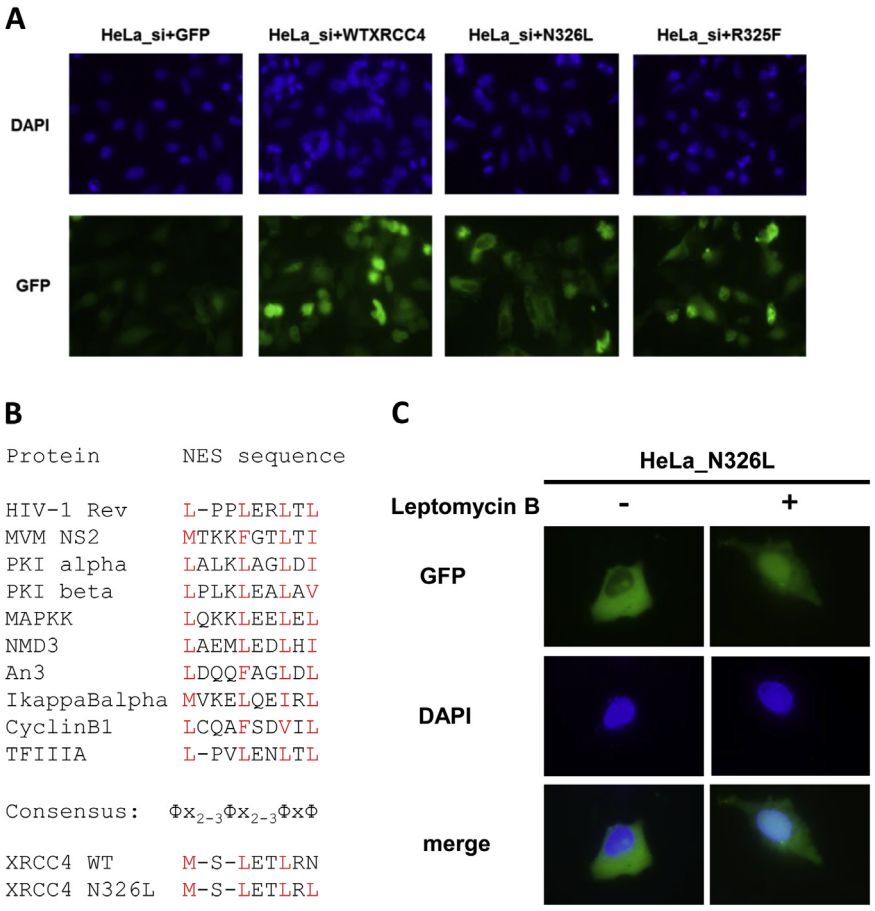

Fig. 2. A: Subcellular localization of wild-type and mutated XRCC4 in HeLa cells. HeLa cells were first transfected with siRNA directed to 3′-UTR of XRCC4 to knockdown endogeneous XRCC4 and then with the expression vectors of wild-type XRCC4, R325F or N326L mutants. B: Alignment of leucine-rich nuclear export signals from example proteins with XRCC4 C-terminus ("Φ" denotes hydrophobic amino acid (L, I, V, F or M), whereas "x" denotes any other amino acid). C: Fluorescence microscopic image of GFP-XRCC4^{N326L} with or without Leptomycin B. HeLa cells sequentially transfected with XRCC4 3′-UTR siRNA and pEGFP- XRCC4^{N326L} were treated with Leptomycin B at the final concentration of 10 ng/ml for 2 h. Nucleus was counter-stained with DAPI.

3.5. Partial restoration of radiosensitivity of N326L by Leptomycin B

As XRCC4^{N326L} was excluded from the nucleus but could accumulate in the nucleus upon Leptomycin B treatment, we next tested whether Leptomycin B could also restore the radiosensitivity of M10-XRCC4^{N326L} (Fig. 3A). Leptomycin B decreased the surviving fraction of M10-GFP and M10-XRCC4^{WT}. This is agreeable with the study by Sasaki et al. [40] showing that Leptomycin B enhanced cell killing of hamster kidney-derived BHK21 cells by radiation, through suppression of potentially lethal damage repair and sublethal damage repair. To the contrary, Leptomycin B increased the surviving fraction of M10-XRCC4^{N326L}. Nevertheless, Leptomycin B-treated M10-XRCC4^{N326L} was more sensitive than Leptomycin B-treated M10-XRCC4^{WT}. Moreover, the surviving fraction of M10-XRCC4^{N326L} did not change by increasing the Leptomycin B concentration from 10 ng/ml to 100 ng/ml. Thus, Leptomycin B could restore the radiosensitivity of M10-XRCC4^{N326L} only partially. These results would indicate that the functional defects of XRCC4^{N326L} might be partially, but not solely, due to its exclusion from nucleus by synthetic nuclear export signal.

3.6. Functional deficiency of other Asn326 mutants

Finally we examined the effects of changing Asn326 into other amino acids, *i.e.*, alanine, aspartic acid and glutamine. Aspartic acid and glutamine were chosen for structural similarity to asparagine. First cDNA expression vector of these mutants were introduced into HeLa cells, in which endogeneous XRCC4 had been knocked down using 3′-UTR siRNA. None of these mutants showed defective nuclear localization function like N326L (Fig. 4A).

Next cDNA expression vector of these mutants were introduced into M10 cells and stable transformant clones were established. These clones were assessed for survival after γ-irradiation through colony formation assay. All of these three mutants, *i.e.*, M10-XRCC4^{N326A}, M10-XRCC4^{N326D} and M10-XRCC4^{N326Q} showed the radiosensitivity between that of the M10-XRCC4^{WT} and M10-XRCC4^{N326L} (Fig. 4B). Thus, small changes in the side chain of Asn326 were found to significantly compromise XRCC4 function.

4. Discussion

In the present study, we noted that the extremely C-terminal region of XRCC4, *i.e.*, XECT region, is highly conserved among a wide variety of vertebrate species. We showed that changing Asn326 therein to other amino acids results in reduced XRCC4 function. N326L mutant, which was created initially, was defective in nuclear localization due to synthetic nuclear export signal. However, Leptomycin B, which restored nuclear localization of XRCC4, could only partially rescue the radiosensitivity of M10-XRCC4^{N326L}. Moreover, other Asn326 mutants, which showed nuclear localization, still showed elevated radiosensitivity. These results collectively indicated that Asn326 would be essential for DSB repair function of

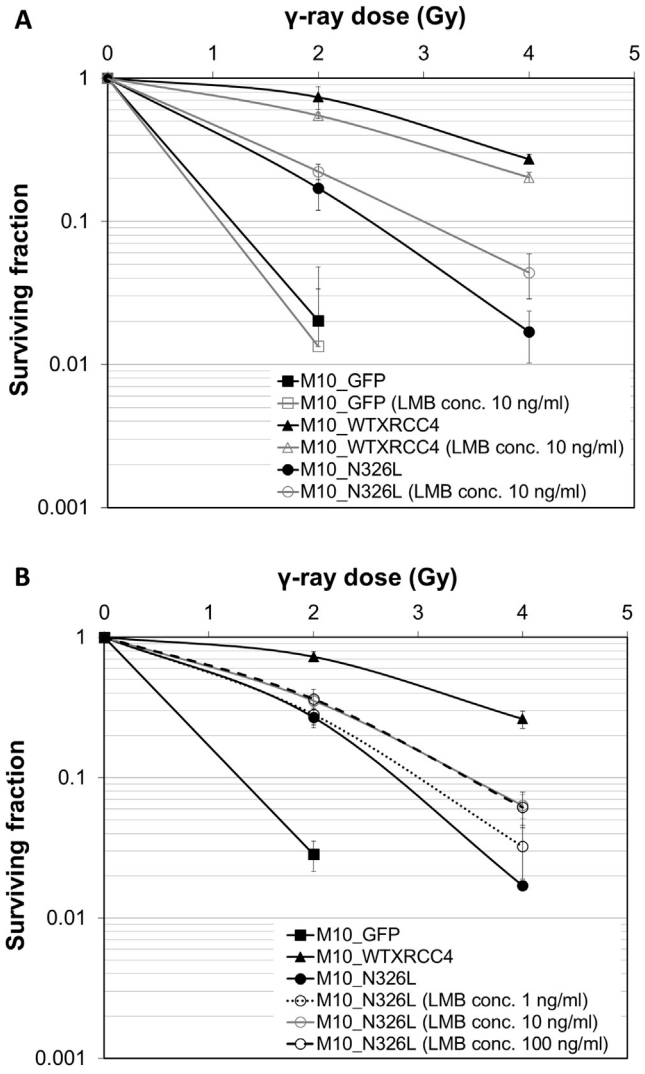

Fig. 3. Effects of Leptomycin B on radiation sensitivity. A: M10-GFP, M10-XRCC4^{WT} and M10-XRCC4^{N326L} cells were treated with 10 ng/ml of Leptomycin B for 2 h prior to γ-irradiation. B: M10-XRCC4^{N326L} cells were treated with 1, 10 and 100 ng/ml of Leptomycin B for 2 h prior to γ-irradiation. The mean of results from more than three repeated experiments is shown with the error bar indicating the standard deviation.

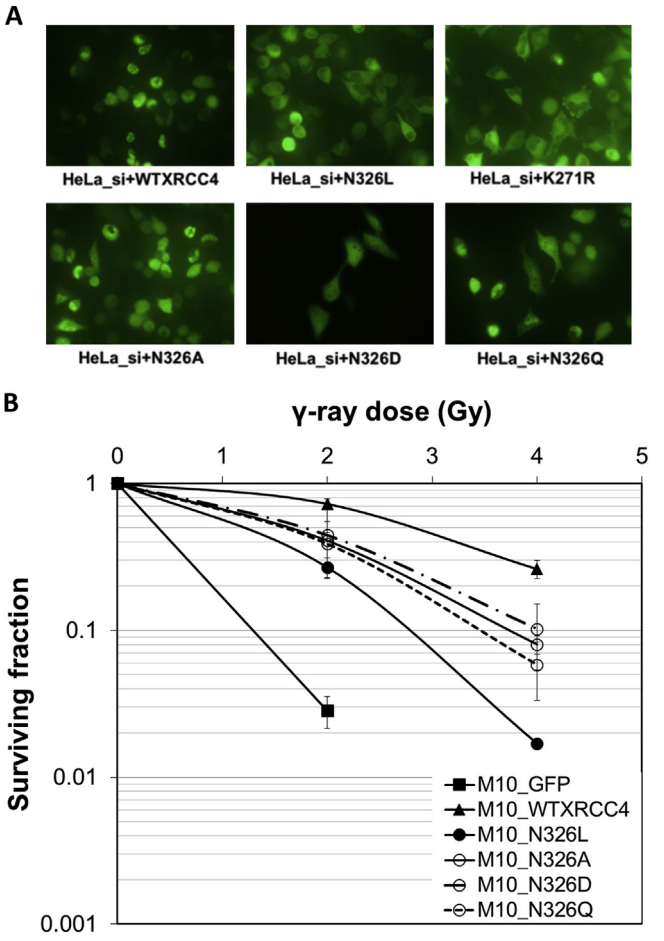

Fig. 4. Functionality of other N326 mutants. A: Subcellular localization of N326 mutants. HeLa cells were first transfected with siRNA directed to 3′-UTR of XRCC4 to knockdown endogeneous XRCC4 and then with the expression vectors of wild-type XRCC4 or N326 mutants. B: Radiosensitivity of Asn326 mutants. Cells were irradiated with 2 Gy and 4 Gy of γ-ray and the cell survival was assessed by colony forming ability in agarose-containing medium. The mean of results from more than three repeated experiments is shown with the error bar indicating the standard deviation.

XRCC4. We would note that Asn326 was conserved among all the species listed in Fig. 1A. We extended the retrieval of XRCC4 amino acid sequences from various vertebrate species and found that Asn326 was almost exclusively conserved (Fig. S2), suggesting its importance in DSB repair in vertebrates.

What is the role of Asn326? Changing Asn326 to either leucine, alanine, aspartic acid or glutamine compromised XRCC4 function in terms of cell survival after irradiation. Asparagine, but not leucine, alanine, aspartic acid and glutamine, undergoes N-glycosylation. However, N-glycosylation is usually seen in extracellular proteins or domains, whereas XRCC4 is nuclear protein. It might be interesting to note that C-terminal regions of other NHEJ proteins are shown to be essential. Point mutation at fourth amino acid (E4120K) from the C-terminus of DNA-PKcs resulted in great decrease in its function [41]. Twelve amino acids at the C-terminus of Ku86 are essential for its interaction with DNA-PKcs [42,43]. Finally, C-terminus of XLF is shown to be essential for interaction with Ku. Considering this, XECT may also be a module for protein–protein interactions [44].

The present study showed the conservation and importance of the extremely C-terminal region of XRCC4, *i.e.*, XECT region. Further studies are required to clarify the role of XECT region and Asn326 therein.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.015.

Transparency document

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