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Lysine 271 but not lysine 210 of XRCC4 is required for the nuclear localization of XRCC4 and DNA ligase IV

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ABSTRACT

XRCC4 and DNA Ligase IV (LIG4) cooperate to join two DNA ends at the final step of DNA double-strand break (DSB) repair through non-homologous end-joining (NHEJ). However, it is not fully understood how these proteins are localized to the nucleus. Here we created XRCC4^{K271R} mutant, as Lys271 lies within the putative nuclear localization signal (NLS), and XRCC4^{K210R} mutant, as Lys210 was reported to undergo SUMOylation, implicated in the nuclear localization of XRCC4. Wild-type and mutated XRCC4 with EGFP tag were introduced into HeLa cell, in which endogenous XRCC4 had been knocked down using siRNA directed to 3′-untranslated region, and tested for the nuclear localization function by fluorescence microscopy. XRCC4^{K271R} was defective in the nuclear localization of itself and LIG4, whereas XRCC4^{K210R} was competent for the nuclear localization with LIG4. To examine DSB repair function, wild-type and mutated XRCC4 were introduced into XRCC4-deficient M10. M10-XRCC4^{K271R}, but not M10-XRCC4^{K210R}, showed significantly reduced surviving fraction after 2 Gy γ-ray irradiation as compared to M10-XRCC4^{WT}. The number of γ-H2AX foci remaining 2 h after 2 Gy γ-ray irradiation was significantly greater in M10-XRCC4^{K271R} than in M10-XRCC4^{WT}, whereas it was only marginally increased in M10-XRCC4^{K210R} as compared to M10-XRCC4^{WT}. The present results collectively indicated that Lys271, but not Lys210, of XRCC4 is required for the nuclear localization of XRCC4 and LIG4 and that the nuclear localizing ability is essential for DSB repair function of XRCC4.

1. Introduction

DNA double-strand break (DSB) is considered the most deleterious type of DNA damage, leading to cell death or tumorigenesis unless repaired properly. Eukaryotic cells repair DSB mainly through two pathways, *i.e.*, homologous recombination (HR) and non-homologous end joining (NHEJ) [1]. NHEJ is considered less precise than HR but is available throughout the cell cycle. NHEJ plays an essential role also in V(D)J recombination in vertebrate immune systems. Six molecules are thought to play pivotal roles in NHEJ in vertebrate: Ku70, Ku86 (also known as Ku80), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV (LIG4) and XLF (also known as Cernunnos) [1,2]. Ku70 and Ku86 first bind to DSB and then recruit DNA-PKcs. LIG4, in cooperation with XRCC4 and XLF, finally joins two DSB ends [1].

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 cell, which belongs to the complementation group 4 of ionizing radiation-sensitive mutants [2]. It was subsequently found that XRCC4 interacts with and stimulates the activity of LIG4 [3,4]. Human XRCC4 consists of 334 or 336 amino acids (because of the alternative splicing inserting 6 nucleotides in the latter, replacing lysine 298 in the former with asparagine-serine-arginine triplet), assembled into N-terminal globular head domain (amino acids 1–115), middle stalk domain

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; D-PBS(−), Ca^{2+}- and Mg^{2+}-free Dulbecco's Phosphate-Buffered Saline; DSB, double-strand break; FBS, fetal bovine serum; NHEJ, non-homologous end-joining; NLS, nuclear localization signal; LIG4, DNA ligase IV; UTR, untranslated region.

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(amino acids 119–203) and C-terminal domain, which is structurally disordered [5–7] (Fig. 1). The stalk domain mediates its homodimerization and interaction with LIG4. The interface of XRCC4 to interact with LIG4 lies between amino acids 173–195. In LIG4, the region linking two BRCT domains, *i.e.*, amino acids 755-782, interacts with XRCC4 [6] and the second BRCT domain is shown to strengthen this interaction [7].

XRCC4 has a putative nuclear localization signal (NLS) sequence, RKRRQR, corresponding to amino acids 270–275 in human XRCC4. In support of the functionality of this putative NLS, Grawunder et al. showed that XRCC4 lacking amino acids 250–300 ($XRCC4^{\Delta 250-300}$) failed to be localized in the nucleus [8]. Girard et al. also showed defective nuclear localization of truncated XRCC4 lacking amino acids 266–334 ($XRCC4^{1-265}$) [9]. There are several lines of evidence that nuclear localization of LIG4 and/or its stability therein depend on the interaction with XRCC4. Bryans et al. showed LIG4 is diminished to an undetectable level in XRCC4-deficient XR-1 cell [10]. Berg et al. showed that XRCC4 is required for efficient import into and stable existence of LIG4 in the nucleus [11]. On the other hand, there are several studies indicating that LIG4 is required for the nuclear localization and the recruitment to DSB of XRCC4. Drouet et al. demonstrated that DNA damage-induced mobilization of XRCC4 as well as LIG4 into the insoluble fraction, containing chromatin and nuclear matrix, was decreased in fibroblasts from a patient of LIG4 syndrome (R278H) [12]. In our recent study, the chromatin binding of XRCC4 was absent in LIG4-disrupted cells derived from human pre-B leukemia cell Nalm-6 [13]. Additionally, Francis et al. very recently demonstrated that XRCC4 existed in the cytoplasm and was absent in the nucleus in LIG4-disrupted Nalm-6 [14]. They also showed that XRCC4 in the nucleus was decreased in fibroblasts from two LIG4 syndrome patients (R278H and R814X, respectively) [14]. Girard et al. identified two tandem NLS, *i.e.*, KKRK and KMKK, which span amino acids 626-629 and 633-636, respectively [9]. LIG4 could be localized to the nucleus in a manner dependent on this NLS, when expressed in XRCC4-deficient XR-1 cell [9]. Co-expression of wild-type XRCC4 with NLS-mutated LIG4 (6 lysine residues changed into threonine) could restore the nuclear localization of LIG4 and co-expression of wild-type LIG4 with $XRCC4^{1-265}$ could restore the nuclear localization of XRCC4. These results collectively indicated that both of LIG4 and XRCC4 have NLS, either one of which might be sufficient for the nuclear localization of the XRCC4-LIG4 complex.

Yurchenko et al. reported that XRCC4 undergoes SUMOylation at Lys210 [15]. $XRCC4^{K210R}$ failed to be localized in the nucleus but conjugating SUMO at C-terminal of $XRCC4^{K210R}$ restored nuclear localization, indicating that SUMOylation at Lys210 is necessary and sufficient for the nuclear localization of XRCC4 [15]. They further showed that $XRCC4^{K210R}$ is devoid of the ability to restore normal radiosensitivity and V(D)J recombination ability to XR-1, whereas SUMO-conjugated $XRCC4^{K210R}$ could restore normal radiosensitivity to XR-1 [15].

Thus, the current knowledge on the mechanisms of nuclear localization of XRCC4 and LIG4 is complicated, necessitating further studies. In this study, we analyzed the roles of Lys271 and Lys210 in the nuclear localization of XRCC4 and LIG4.

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 and integrated into p3XFLAG-CMV-10 vector (Sigma–Aldrich; St. Louis, MO, USA) [16]. To express XRCC4 as a fusion protein with enhanced green fluorescent protein (EGFP), XRCC4 cDNA was excised from p3XFLAG-CMV-10 vector using restriction enzymes *Eco*RI and *Xho*I and inserted into pEGFP-C1 vector (Clontech; Mountain View, CA, USA) using *Eco*RI and *Sal*I. Point mutations were introduced using PrimeSTAR Mutagenesis Kit (Takara Bio; Otsu, Shiga, Japan). Sequences of PCR primers for mutagenesis are as follows (underlined nucleotides correspond to mutated amino acids: K210R–F, GAC ATC AGG CAA GAA GGG GAA ACT GCA; K210R-R, TTC TTG CCT GAT GTC CTT TTC TCG TTC; K271R–F, AGT AGA AGG AGG AGA CAG CGA ATG CAA; K271R-R, TCT CCT CCT TCT ACT TGG TGC AAT ATC. Correctness of the sequence of entire XRCC4 open reading frame was verified for all the constructs. The vector expressing human LIG4 was constructed as described recently [13].

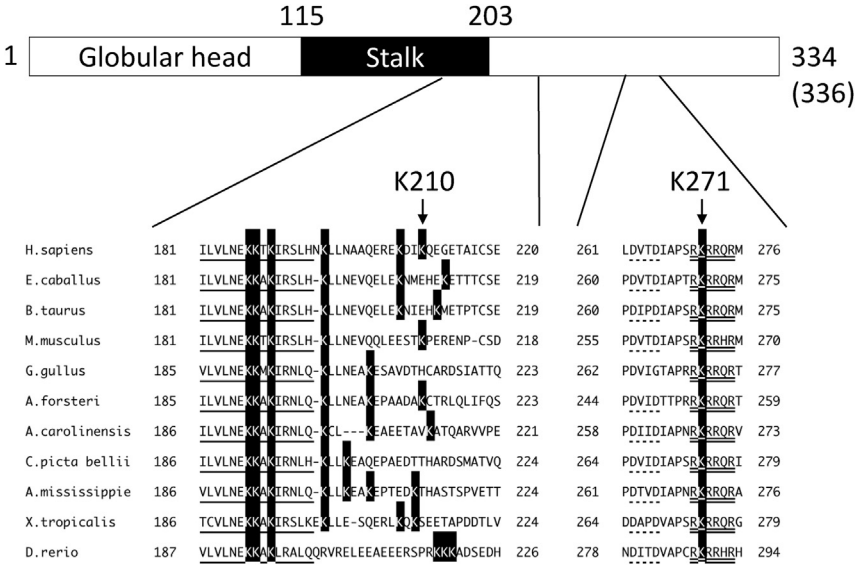


Fig. 1. Schematic presentation of XRCC4 structure and conservation of amino acid sequence surrounding Lys210 and Lys271. Single underlines indicate the LIG4 binding region. Dotted underlines indicate the caspase cleavage motif. Double underlines indicate the putative nuclear localization signal (NLS). Lysines are highlighted by black and white reversal.

2.2. Cell culture

Human cervical carcinoma cell line HeLa was cultured in DMEM/Ham's F-12 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified atmosphere containing 5% CO_2. To knockdown the endogenous XRCC4 in HeLa cell, small interfering RNA (siRNA) targeting 3′-untranslated region (UTR) was applied [17]. The sequences of 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. As the control, scrambled duplex of the following sequence was used: 5′- UCU CUA UUA UUA GUU CUC UdTdT -3′, 5′- AGA GAA CUA AUA AUA GAG AdAdA -3′. Oligonucleotides were synthesized by Japan Bioservice (Saitama, Japan). The siRNA was transfected into HeLa cell using Lipofectamine RNAi MAX (Invitrogen; Carlsbad, CA, USA) and, 24 h later, wild-type or mutated XRCC4 cDNA was transfected using Lipofectamine 2000 (Invitrogen). Where indicated, LIG4 cDNA [13] in pCMV10-3XFLAG vector was transfected together with XRCC4.

Murine leukemia L5178Y-drived, XRCC4-deficient cell line M10 was obtained from RIKEN cell bank (Tsukuba, Ibaraki, Japan) with the permission of Dr. Koki Sato (National Institute of Radiological Sciences and Kinki University). M10 cell harbor mutation in XRCC4 gene (c.A370T, p.R124X), thus are defective in DSB repair and radiosensitive [18,19]. M10 cell and its transformants were cultured in RPMI1640 medium (Nacalai Tesque; Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone; Logan, UT, USA), 100 units/ml penicillin, 100 μg/ml streptomycin and 10 μM β-mercaptoethanol at 37 °C in humidified atmosphere containing 5% CO_2 [15]. Wild-type or mutated XRCC4 cDNA was introduced into M10 cell using Neon Transfection system (Invitrogen). Two days after the transfection, cells were plated in RPMI1640 medium supplemented with 15% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μM β-mercaptoethanol, 0.8 mg/ml G418 and 0.2% agarose (Becton, Dickinson and Company; Franklin Lakes, NJ, USA). Two weeks after the plating, visible colonies were picked up and expanded to obtain stably transformed clones.

2.3. Assessment of cellular radiosensitivity

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). The cellular radiosensitivity was assessed in terms of colony forming ability in soft agarose as described [20]. One thousand cells were suspended in 12 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 divided into three plastic dish of 60 mm in diameter. After culturing for 10–12 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 by the plating efficiency of unirradiated control. One-sided Welch's t-test was applied for the statistical analyses.

2.4. Antibodies, western blotting, immunoprecipitation and immunostaining

Anti-XRCC4 rabbit polyclonal antibody was described earlier [15]. Following commercial antibodies were used: anti-LIG4 rabbit polyclonal antibody (ab80514; Abcam; Bristol, UK), anti-γH2AX rabbit polyclonal antibody (#7-164; Millipore; Temecula, CA, USA), anti-PCNA rabbit polyclonal antibody (FL261; Santa Cruz Biotechnology; Santa Cruz, CA, USA) and anti-rabbit immunoglobulin swine polyclonal antibody conjugated with horseradish peroxidase (P0447; DAKO; Glostrup, Denmark).

Procedures of Western blotting followed our earlier publications [13,16].

For immunoprecipitation, 10^7 cells were suspended in 1 ml of IP Lysis Buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.5% Triton X-100 supplemented with cocktail of proteases inhibitors and phosphatase inhibitors) and, after 30 min on ice, were centrifuged at 15,000 rpm for 7 min. The supernatant was mixed with anti-FLAG M2 affinity gel (Sigma–Aldrich). The reaction tubes were kept at 4 °C overnight with gentle agitation by rotation. The beads were precipitated by centrifugation at 10,000 rpm for 1 min and washed 3 times with 1 ml of IP Lysis Buffer. Proteins were eluted from the gel by 2XSDS-PAGE sample buffer with heating in boiling water and subjected to Western blotting.

For immunostaining, HeLa cells were grown on the glass-bottomed dish (AGC Techno Glass; Tokyo, Japan). M10 cells, grown in suspension, were attached on a slide glass (Matsunami Glass; Osaka, Japan) by centrifugation at 100 g for 5 min. Cells were fixed with 4% paraformaldehyde in Ca^{2+}- and Mg^{2+}-free Dulbecco's Phosphate-Buffered Saline (D-PBS(−)) and permeabilized with 0.5% Triton X-100 in D-PBS(−) for 30 min followed by dehydration with 70% ethanol and rehydration with D-PBS(−). Slides were incubated with D-PBS(−) containing 5% bovine serum albumin (BSA) without immunoglobulin and then with the primary antibodies. After rinsing twice with D-PBS(−) containing 0.05% Tween 20 (PBST), slides were incubated in the dark with Alexa Flour 594-conjugated secondary antibody (Abcam) diluted with PBST. After rinsing twice with PBST, slides were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescence was observed with an inverted fluorescence microscope, IX71 (Olympus; Tokyo, Japan). One-sided Welch's t-test was applied for the statistical analyses.

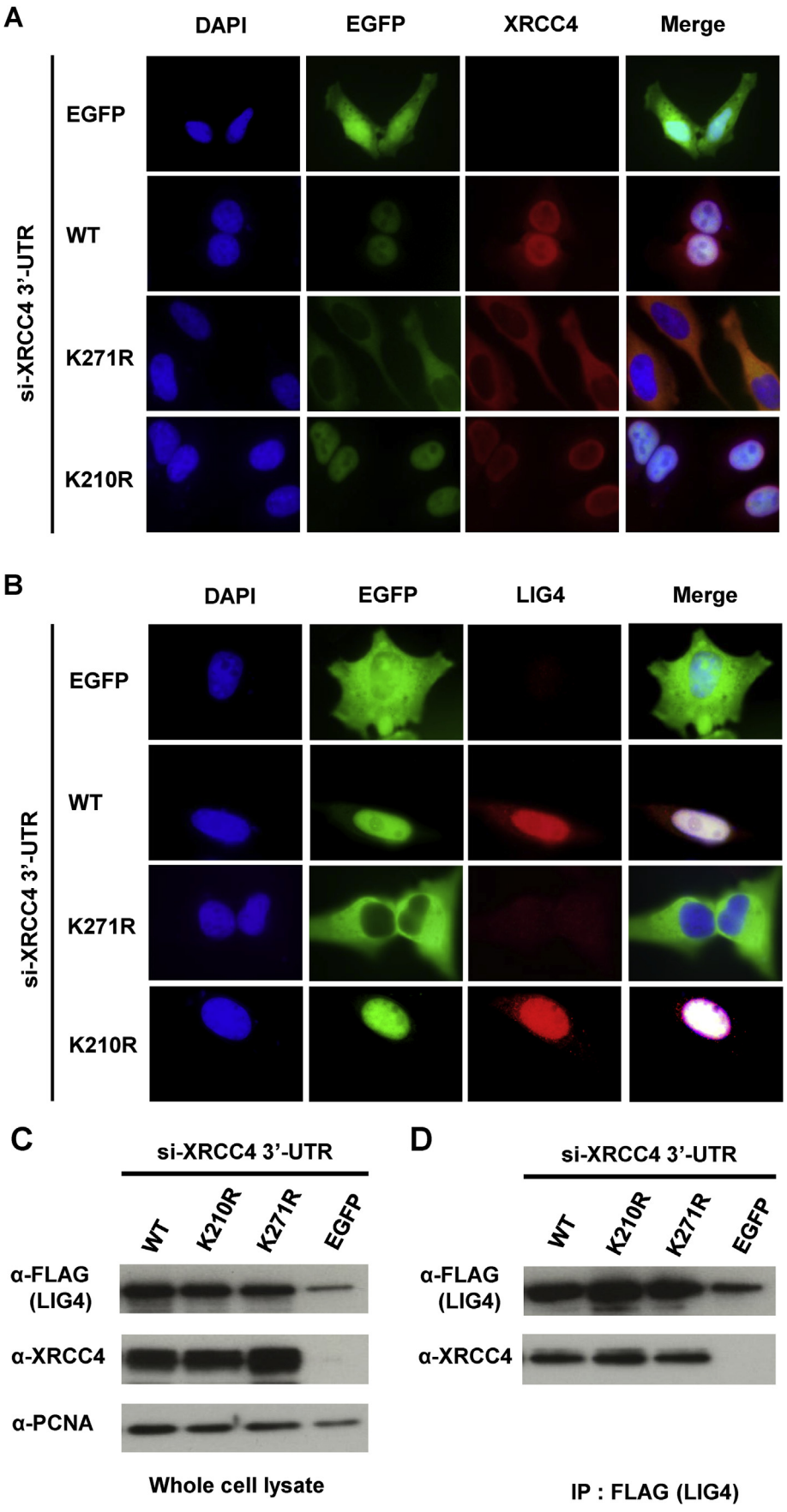
3. Results

3.1. $XRCC4^{K271R}$ mutant is defective in the nuclear localization of itself and LIG4

The sequence RKRRQR, spanning amino acids 270–275 of human XRCC4, is considered NLS. This sequence is highly conserved among wide range of vertebrate species (Fig. 1). It was shown that deleting amino acids 250–300 or 266–334 of human XRCC4, containing this putative NLS, resulted in defective nuclear localization [8,9]. We sought to examine more precisely the functionality of this putative NLS by creating a point mutant. We chose lysine at the second position, *i.e.*, Lys271 in human XRCC4, and changed it into arginine to preserve positive charge, creating $XRCC4^{K271R}$ mutant.

To examine the subcellular localization, wild-type or mutated XRCC4 was expressed in HeLa cell in fusion with EGFP using pEGFP-C1 vector and observed by means of green fluorescence and immunostaining (Fig. 2A). HeLa cell has endogenous XRCC4, which may dimerize with and escort exogenous XRCC4 into the nucleus. Hence we thought it necessary to eliminate endogenous XRCC4. We synthesized two siRNA duplexes targeted to 5′- and 3′-UTR, respectively, so that it would not affect the expression of exogenous XRCC4. As it was found that 3′-UTR siRNA, but not 5′-UTR siRNA, could effectively decrease endogenous XRCC4, 3′-UTR siRNA was used below.

In cells transfected with XRCC4 3′-UTR siRNA and control EGFP, EGFP signal was seen throughout the cell, including the nucleus (Fig. 2A, first row from the top, second column from the left). Such distribution of NLS-less GFP was also observed in earlier studies [8,9], possibly due to the diffusion of GFP into the nucleus because of small size. It would be noted that immunofluorescence using


A
DAPI
EGFP
XRCC4
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si-XRCC4 3'-UTR
EGFP
WT
K271R
K210R
B
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si-XRCC4 3'-UTR
EGFP
WT
K271R
K210R
C
si-XRCC4 3'-UTR
WT
K210R
K271R
EGFP
α-FLAG (LIG4)
α-XRCC4
α-PCNA
Whole cell lysate
D
si-XRCC4 3'-UTR
WT
K210R
K271R
EGFP
α-FLAG (LIG4)
α-XRCC4
IP : FLAG (LIG4)

anti-XRCC4 antibody was not seen in these cells (first row, third column), indicating the specificity of this antibody and successful knockdown of endogenous XRCC4. XRCC4^{WT} was localized to the nucleus, but XRCC4^{K271R} was excluded from the nucleus (second and third rows, second and third columns). These results indicated that this putative NLS is really functional and that K271R mutation disrupts its functionality.

We then co-transfected LIG4 with XRCC4 and examined their localization (Fig. 2B). LIG4 was barely detectable in cells co-transfected with XRCC4 3′-UTR siRNA and control EGFP (first row, third column). In cells transfected with XRCC4^{WT} cDNA, strong nuclear staining with LIG4 antibody was observed (second row, third column). This is agreeable with earlier studies showing that XRCC4 is required for the stability of LIG4 [10]. In XRCC4^{K271R} transfectant, anti-LIG4 antibody exhibited weak, homogenous staining throughout nucleus and cytoplasm (third row, third column). These results indicated that the XRCC4 NLS containing Lys271 is essential for the nuclear localization of LIG4.

The expression level of XRCC4 and LIG4 in the above condition was examined by Western blotting analysis (Fig. 2C). The results showed equivalent expression of XRCC4 and LIG4 in cells transfected with XRCC4^{WT}, XRCC4^{K210R} and XRCC4^{K271R}. LIG4 expression level was significantly decreased in cells transfected with control EGFP. The interaction between XRCC4 and LIG4 was examined by immunoprecipitation using anti-FLAG affinity gel (as LIG4 was tagged with 3XFLAG sequence) followed by Western blotting analysis (Fig. 2D). XRCC4^{WT}, XRCC4^{K210R} and XRCC4^{K271R} were co-precipitated at equivalent amount, indicating that the ability of XRCC4 to interact with LIG4 was not abrogated by K271R mutation.

3.2. *XRCC4^{K210R} mutant is competent for the nuclear localization of itself and LIG4*

Having established that RKRRQR sequence in human XRCC4 is NLS and that changing Lys271 into arginine compromised its function, we next examined the effect of changing Lys210 into arginine in the nuclear localization of XRCC4 and LIG4. XRCC4^{K210R} was localized to the nucleus like XRCC4^{WT} (Fig. 2A, fourth row, second and third column). When LIG4 was co-transfected with XRCC4^{K210R}, LIG4 was localized to the nucleus (Fig. 2B, fourth row, third column). These results indicated that XRCC4^{K210R} mutant has the ability to localize itself and LIG4 in the nucleus.

3.3. *XRCC4^{K271R} is deficient in DSB repair function but XRCC4^{K210R} retains mostly normal DSB repair function*

To evaluate the DSB repair function, wild-type or mutated XRCC4 was introduced into M10, murine leukemia-derived cell lacking XRCC4, and stable transformants were established. Western blotting analyses indicated that XRCC4^{WT}, XRCC4^{K271R} and XRCC4^{K210R} were expressed at an equivalent level (Fig. 3A). These transformants were tested for the surviving fraction after 2 Gy irradiation (SF2) by colony formation assay and the number of γ−H2AX foci remaining 2 h after 2 Gy γ-ray irradiation by immunofluorescence.

SF2 of M10-XRCC4^{K271R} was significantly lower than that of M10-XRCC4^{WT} and that of M10-XRCC4^{K210R} (Fig. 3B, $p < 0.001$ and $p = 0.013$, respectively, in one-sided Welch's t-test). On the other hand, SF2 of M10-XRCC4^{K210R} was not different from that of M10-XRCC4^{WT} (Fig. 3B, $p = 0.20$ in one-sided Welch's t-test).

The number of γ−H2AX foci remaining 2 h after 2 Gy γ-ray irradiation in M10-XRCC4^{K271R} was significantly greater than that in M10-XRCC4^{WT} (Fig. 3C and D, $p < 0.001$ in one-sided Welch's test). On the other hand, the number of γ-H2AX foci in M10-XRCC4^{K210R} was only marginally greater than that in M10-XRCC4^{WT} (Fig. 3C and D, $p = 0.035$ in one-sided Welch's t-test).

These results indicated that M10-XRCC4^{K271R} is deficient in DSB repair function and M10-XRCC4^{K210R} retains mostly normal function, although some slight defect in M10-XRCC4^{K210R} cannot be excluded. This is consistent with defective nuclear localization of XRCC4^{K271R} and competence of XRCC4^{K210R} for nuclear localization.

4. Discussion

It has been reported that XRCC4 is required for the nuclear localization of LIG4 [11]. To the contrary, it has been also reported that LIG4 is required for the nuclear localization of XRCC4 [12]. Furthermore, there is a study showing that both of XRCC4 and LIG4 have NLS, either one of which might be sufficient for the nuclear localization of the XRCC4-LIG4 complex [9]. The present results indicated that XRCC4 NLS, containing Lys271, is essential for the nuclear localization of itself and LIG4 as well (Fig. 2).

Nuclear localization would have two requirements: import into the nucleus and stability in the nucleus. Two models can be considered for the nuclear import of XRCC4 and LIG4 (Fig. 4). In the first model, XRCC4 and LIG4 are imported to the nucleus together by means of XRCC4 NLS or LIG4 NLS (Fig. 4A). In the second model, XRCC4 and LIG4 are imported separately into the nucleus and associate with each other in the nucleus (Fig. 4B). According to the first model, XRCC4 and LIG4 would have been imported into the nucleus by means of LIG4 NLS, even if XRCC4 NLS was dysfunctional (Fig. 4C). According to the second model, LIG4 should have been imported into the nucleus in XRCC4^{K271R} cells but would be unstable because of the absence of XRCC4 in nucleus (Fig. 4D). Thus, the present results would be more compatible with the second model.

We also demonstrated that XRCC4^{K271R} is deficient in DSB repair function as measured by radiosensitivity and γ−H2AX foci (Fig. 3). In the earlier study by Grawunder et al., XRCC4$^{\Delta 250-300}$, lacking NLS, fully rescued radiosensitivity and V(D)J recombination defects of XRCC4-deficient XR-1 cell [8,21]. Although XRCC4$^{\Delta 250-300}$ was observed in the nucleus as well as in the cytoplasm in their study, XRCC4^{K271R} was excluded from the nucleus in the present study. It was observed, nevertheless, that M10-XRCC4^{K271R} was much less radiosensitive than M10-EGFP, indicating residual DSB repair function of XRCC4^{K271R}. It might be possible that small portion of XRCC4 could be imported into the nucleus due to diffusion or LIG4 NLS.

The present study also showed that XRCC4^{K210R} could be localized to the nucleus together with LIG4 (Fig. 2). XRCC4^{K210R} exhibited mostly normal DSB repair function as measured by radiosensitivity and γ−H2AX foci, although some slight defect in M10-XRCC4^{K210R} cannot be excluded (Fig. 3). These results are in contrast to the earlier study by Yurchenko et al. showing that

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 endogenous XRCC4 and then with the expression vectors of XRCC4^{WT}, XRCC4^{K271R} or XRCC4^{K210R} mutants, in fusion with EGFP. XRCC4 was visualized by means of fluorescence of EGFP and immunostaining using anti-XRCC4 antibody and fluorescent secondary antibody. Nucleus was counter-stained with DAPI. B: Ability of wild-type and mutated XRCC4 in driving nuclear localization of LIG4. Following transfection of HeLa cells with XRCC4 3′-UTR siRNA, the expression vectors of XRCC4 (as indicated) and LIG4 were introduced. DAPI staining of nucleus, fluorescence of EGFP and immunostaining using anti-LIG4 antibody are shown. C: Expression level of EGFP-XRCC4 and FLAG-LIG4 in the same condition as B examined by Western blotting analysis. PCNA is shown as the loading control. D: Interaction between EGFP-XRCC4 and FLAG-LIG4. The whole cell lysate shown in C was immunoprecipitated using anti-FLAG affinity gel and the precipitate was examined by Western blotting analysis.

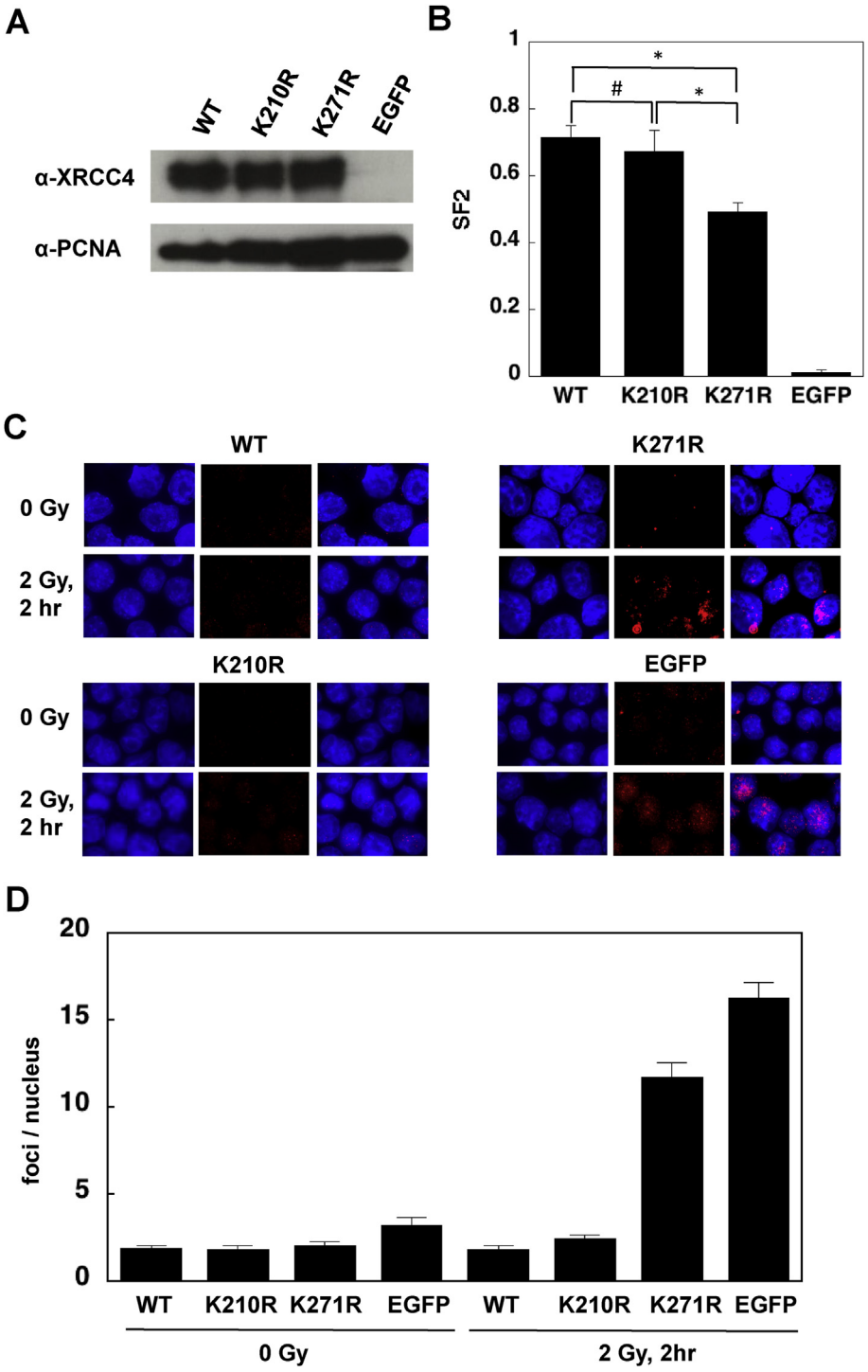

Fig. 3. A: Western blotting analyses of XRCC4 expression in M10-XRCC4^{WT}, M10-XRCC4^{K271R}, M10-XRCC4^{K210R} and M10-EGFP cells. PCNA is also shown as the loading control. B: Surviving fraction after 2 Gy irradiation (SF2) of M10-XRCC4^{WT}, M10-XRCC4^{K271R}, M10-XRCC4^{K210R} and M10-EGFP cells. The mean of results from three repeated experiments is shown with the error bar indicating the standard deviation. C: Immunostaining of γ−H2AX in M10-XRCC4^{WT}, M10-XRCC4^{K271R}, M10-XRCC4^{K210R} and M10-EGFP cells harvested 2 h after exposure to 2 Gy of γ−ray. Nucleus was counter-stained with DAPI. D: Counts of γ−H2AX foci. The number of γ−H2AX foci was counted in more than 100 nuclei and the average is shown with the standard error of means indicated by the error bar.

XRCC4^{K210R} was devoid of nuclear localization and ability to rescue radiosensitivity and V(D)J recombination defects of XRCC4-deficient Chinese hamster ovary cell XR-1 [15]. It might be noted that the amino acid sequence corresponding to the region surrounding Lys210 in human XRCC4 is divergent even among mammalian species (Fig. 1). Although the exact reason for the apparent discrepancy is currently unclear, the requirement for SUMOylation in XRCC4 nuclear localization might depend on species. Alternatively, there might be other SUMOylation site(s), which can substitute for Lys210.

In conclusion, the present results demonstrated that Lys271, but not Lys210, of XRCC4 is required for the nuclear localization of XRCC4 and LIG4 and that the nuclear localizing ability is essential for DSB repair function of XRCC4.

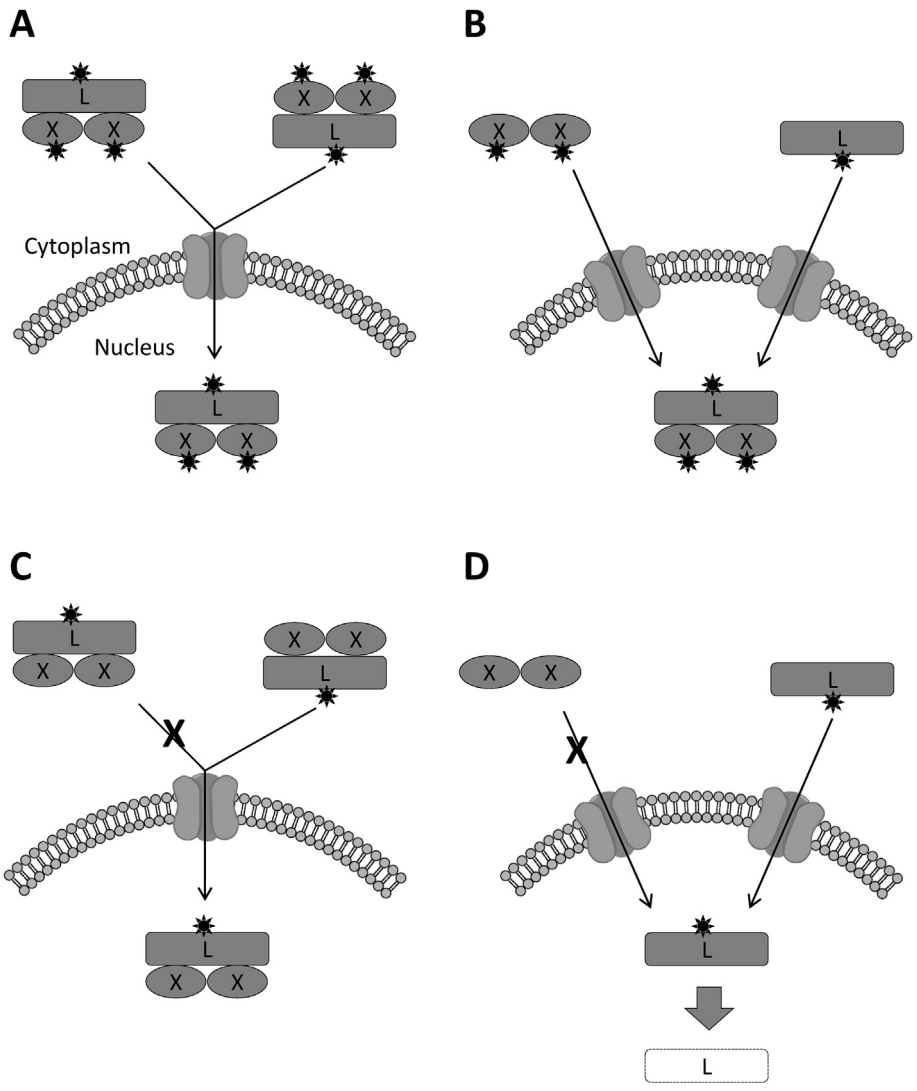

Fig. 4. Possible models for the nuclear localization of XRCC4 and LIG4. Ellipses (X) and rectangles (L) stand for XRCC4 and LIG4, respectively. Asteroids indicate NLS. A: XRCC4 and LIG4 are imported into the nucleus together by means of XRCC4 NLS or LIG4 NLS. B: XRCC4 and LIG4 are imported into the nucleus separately and associate with each other in the nucleus. C, D: Expected behavior of NLS-deficient XRCC4 and LIG4, based on the model shown in A and B, respectively.

Conflict of interest

None.

Acknowledgments

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