

# XRCC4's interaction with XLF is required for coding (but not signal) end joining

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## ABSTRACT

**XRCC4 and XLF are structurally related proteins important for DNA Ligase IV function. XRCC4 forms a tight complex with DNA Ligase IV while XLF interacts directly with XRCC4. Both XRCC4 and XLF form homodimers that can polymerize as heterotypic filaments independently of DNA Ligase IV. Emerging structural and *in vitro* biochemical data suggest that XRCC4 and XLF together generate a filamentous structure that promotes bridging between DNA molecules. Here, we show that ablating XRCC4's affinity for XLF results in DNA repair deficits including a surprising deficit in VDJ coding, but not signal end joining. These data are consistent with a model whereby XRCC4/XLF complexes hold DNA ends together—stringently required for coding end joining, but dispensable for signal end joining. Finally, DNA-PK phosphorylation of XRCC4/XLF complexes disrupt DNA bridging *in vitro*, suggesting a regulatory role for DNA-PK's phosphorylation of XRCC4/XLF complexes.**

## INTRODUCTION

Developing lymphocytes usurp the ubiquitously expressed 'classical' non-homologous end joining (c-NHEJ) pathway to resolve DNA double-strand breaks (DSBs) introduced by the RAG endonuclease during the process of VDJ recombination that assembles functional

antigen-binding receptor genes from component gene segments (1). The RAG complex introduces DSBs immediately adjacent to immune receptor gene segments resulting in blunt phosphorylated signal ends and covalently sealed hair-pinned coding termini which are joined by c-NHEJ (2). Coding and signal ends are joined at very different rates, rapid for coding ends while slower for signal ends (3). This is likely explained by the fact that the RAG complex remains tightly associated with signal ends after cleavage in a post cleavage complex, whereas coding ends are released from this post cleavage complex and need to be brought together again for repair (4).

Seven c-NHEJ components are required for VDJ recombination: Artemis, KU70, KU86, DNA-PKcs, XRCC4, XLF and DNA Ligase IV (5). Although all seven are required for efficient coding end joining, the dependence of signal end joining on these c-NHEJ factors is less absolute. Artemis is not required for signal end joining; a reflection of its specific role in opening the sealed hair-pinned termini of cleaved coding ends (6). The dependence of signal end joining on DNA-PKcs is variable. Artemis's endonuclease activity requires physical interaction with DNA-PKcs as well as DNA-PK's enzymatic activity. Because Artemis is dispensable for signal end joining it is reasonable to assume that signal end joining might also progress normally in the absence of DNA-PKcs (6). Indeed, signal end joining proceeds fairly efficiently in developing murine lymphocytes (~10–50% of wild-type levels) and in some murine and one human cell line deficient in DNA-PKcs (7,8). However, DNA-PKcs deficiency in other species (hamster, horse, dog) results in a more severe reduction

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in signal end joining (~30- to 1000-fold reduced) (9,10,11). The RAG complex (and possibly other factors) may help maintain synapsis of signal ends if other factors important for synapsis (like DNA-PKcs or other non-core NHEJ factors that may vary in expression levels in different cell lines or species) are lacking or limiting, perhaps partially explaining the variable asymmetry of effects on coding versus signal end joining in the absence of DNA-PKcs. Although the severity of VDJ deficits differs in cells deficient in any of the other five core c-NHEJ components [XLF < KU protein < XRCC4 or DNA Ligase IV], the relative effect on signal versus coding end joining is similar in each (12). In contrast, cells deficient in factors that clearly promote c-NHEJ, but are not absolutely required for c-NHEJ (ATM, MRN, 53BP1) have only modest VDJ recombination deficits which are remarkably specific to coding end resolution (13–15); it has been suggested that the function of ATM (and perhaps other factors upstream or downstream of ATM) in c-NHEJ is to promote bridging of DNA ends.

Thus, an emerging consensus suggests that asymmetry of coding and signal end joining is a reflection not only of a requirement to open hair-pinned coding ends, but also on a more stringent requirement for synapsis (by repair machinery) to promote efficient coding end joining. Moreover, the variability in the proficiency of signal end joining in different DNA-PKcs deficient cell lines and species might be explained by a more efficient ability to maintain synapsis of signal ends in the absence of DNA-PKcs in certain species and or cell lines (16,17).

XLF, the most recently discovered c-NHEJ factor interacts with the DNA Ligase IV complex via direct interaction with XRCC4 (18,19). A complex of XRCC4, XLF and DNA Ligase IV performs the final ligation step in classical NHEJ. Although XRCC4 and XLF share little primary sequence homology, secondary structure predictions and later crystallographic analyses demonstrated that XLF and XRCC4 are structurally highly related (20,21). Both XRCC4 and XLF form stable homodimers that can associate to form XLF/XRCC4 oligomers (22). Both also bind DNA in a sequence non-specific and concentration-dependent manner with higher affinities as the length of the DNA substrate is increased (23,24). XRCC4's interaction with Ligase IV precludes XRCC4 homo-tetramerization (22,25,26). One DNA Ligase IV molecule interacts with the tail region of one XRCC4 dimer and XLF interacts with XRCC4 but not DNA Ligase IV (26–29). XLF interacts with this complex directly through the XRCC4 N-terminal head domain (21). XLF stimulates ligation of non-cohesive DNA ends, promotes the readenylation of DNA Ligase IV, and gap filling by pol  $\mu$  and pol  $\lambda$  (30–32). Unlike the role of XRCC4 in promoting Ligase IV function which is at least partially understood, the mechanistic basis of how XLF functions to promote DNA Ligase IV's function is largely unknown.

Mutational analysis has revealed a potential interaction interface between XRCC4 and XLF involving the head domains of both molecules (21). These data suggest a stacked head to head interaction between XLF and XRCC4. Small Angle X-Ray Scattering analyses and

new crystallographic data (22,33–37) define XRCC4/XLF filaments that form via the head to head interaction of XRCC4 and XLF; *in vitro*, these XRCC4/XLF complexes promote bridging between DNA molecules. Here, we characterize the functional consequence in living cells of ablating XRCC4's interaction with XLF. Additionally, we explore (both *in vitro* and in living cells) the functional consequence of DNA-PK phosphorylation of XRCC4/XLF complexes.

## MATERIALS AND METHODS

### Plasmids and cell strains

Wild-type and mutant XLF and XRCC4 cDNAs were cloned into the pEF plasmid that provides expression by the EF1 $\alpha$  promoter and also contains the neomycin resistance gene (Invitrogen, Carlsbad, CA, USA). Cell lines utilized in this study include the CHO mutant cell line XR-1 that lacks XRCC4 expression [generous gift of Tom Stamato], wild-type CHO cell line AA8, and Phoenix HEK293 cells [generous gift of Dr Justin McCormick]. XR-1 cells were maintained in  $\alpha$ MEM (Gibco; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum or supplemented calf serum and 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin. XR-1 cells stably expressing wild-type and mutant forms of human XRCC4 were maintained in above media containing 800  $\mu$ g/ml G418. Stable transfectants coexpressing XRCC4 and GFP tagged DNA-PKcs were maintained in above medium along with 800  $\mu$ g/ml G418 and 5  $\mu$ g/ml blasticidin.

### Cell transfections

Ten micrograms of pEF plasmid DNA expressing wild-type or mutant XRCC4 were linearized by PvuI restriction and transfected into XR-1 or AA8 cells. Transfections were performed in 60 mm diameter dishes with the Fugene6 transfection reagent (Roche Molecular Biochemicals; Indianapolis, IN) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were plated into selection media containing 1600  $\mu$ g/ml G418. Independently isolated clones were screened for XRCC4 expression by immunoblot analysis.

XR-1 transfectants expressing wild-type or 9Xala murine XRCC4 have been described previously (38). XLF expression vectors encoding wild-type or 6Xala XLF have been described previously (39). To over-express wild-type or 6Xala XLF in XR-1 cells expressing the 9Xala XRCC4 mutant, 40  $\mu$ g expression plasmid was transfected into the 9Xala transfectant as described above. Forty-eight hours later cells were placed under hygromycin (400  $\mu$ g/ml) selection and stable clones isolated as described above. XLF expression was assessed by immunoblotting.

### Immunoblot analyses

Antibodies utilized in this study include a polyclonal rabbit anti-XRCC4 reagent (Abcam; Cambridge, MA, USA), a polyclonal anti-XLF reagent (Abcam),

a monoclonal anti-V5 reagent (Sigma, St Louis, MO, USA) and phosphospecific antibodies to pS260 and pS318 in XRCC4, raised in sheep against the following phosphopeptides: Ser<sup>260</sup>: SISSLDVTD and Ser<sup>318</sup>: AEN MSLETLR (phosphoserines underlined). Phosphospecific antibodies were affinity purified as described previously (40). Phospho-specific reagents were characterized using site-specific XRCC4 mutant proteins described previously (38).

Whole-cell extracts were obtained by re-suspending cell pellets in solubilization buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 5 mM manganese chloride, 50 mM sodium fluoride, 2 mg/ml DNAase I and protease inhibitor cocktail (Roche Molecular Biochemicals; Indianapolis, IN, USA). For human XRCC4 or human XLF transfectants, 25 µg of each cell extract was electrophoresed on an 8% SDS-PAGE gel and transferred to PVDF membranes. Membranes were probed with either rabbit polyclonal antibody to XRC C4 or XLF. For murine XRCC4 (or 9Xala XRCC4) transfectants, a monoclonal V5 antibody was used as the primary antibody. Anti-rabbit or anti-mouse HRP were used as secondary antibodies and membranes were exposed to chemiluminescent substrate to visualize XRCC4 or XLF.

#### VDJ recombination assays

Extrachromosomal VDJ recombination assays were performed utilizing a coding joint substrate [pJH290] and signal joint substrate [pJH201] as described previously (41). Briefly, XR-1 cells were transiently transfected with 1 µg substrate, 3 µg wild-type or mutant forms of XRCC4 or pEF1 vector, and 3 µg each of RAG1 and RAG2 using the Fugene6 transfection reagent. Forty-eight hours after transfection, substrate plasmids were isolated by alkaline lysis and subjected to DpnI digestion for 1 h. DpnI-digested DNA was transformed into competent DH5α cells (Invitrogen; Carlsbad, CA, USA) according to manufacturers' instructions. Transformed cells were spread onto LB Agar plates containing 100 µg/ml ampicillin only or with 100 µg/ml ampicillin and 22 µg/ml chloramphenicol.

#### Assessment of radiosensitivity and drug sensitivities

To determine sensitivity to ionizing radiation (IR), 4000 cells from each of the XRCC4 wild-type and mutant clones were harvested and treated with various doses of IR in serum free media, using a <sup>60</sup>Co source. Immediately after irradiation, cells were plated back into 100 cm<sup>2</sup> dishes containing αMEM supplemented with 10% FBS. For zeocin resistance assays, 100 cells of each AA8 or XR-1 transfectant were plated in 60 cm<sup>2</sup> dishes containing the appropriate culture media (without selection agents) supplemented with the indicated doses of zeocin. After 7 days, colonies were fixed and stained with crystal violet to establish relative survival.

#### DNA bridging assay

The DNA bridging assay is described by Andres *et al.* (35). The one-end biotinylated 1000 bp DNA substrate

was prepared by PCR using Phusion DNA polymerase, primers: 5'-Biotin GAGTTTTATCGCTTCCAT GAC and 5'-AATTTATCCTCAAGTAAGGGGC and PhiX174 DNA as template. The 500 bp DNA substrate was similarly prepared using primers 5'-GAGTTTTATC GCTTCCATGAC and 5'-CAGAAAATCGAAATCATC TTC. PCR products were purified by agarose gel electrophoresis and QIAquick Gel Extraction Kit (QIAGEN) and stored in 10 mM Tris pH 8, 1 mM EDTA. Magnetic streptavidin-coated beads (Dynabeads M-280 Streptavidin, Invitrogen) were first passivated by washing the bead suspension three times with one volume of binding buffer (20 mM HEPES pH 8, 75 mM KCl, 0.5 mM EDTA, 1 mM DTT, 5% glycerol, 400 µg/ml acetylated BSA) and finally resuspended in the same volume of binding buffer. For each reaction, 200 ng of end biotinylated 1000 bp DNA were added to 10 µl of passivated bead suspension and incubated for 5 min at room temperature (>90% attachment). Next, 200 ng of 500 bp DNA fragment were added before addition of the XRCC4, XLF (each at 2 µM) in a total volume of 40 µl in binding buffer supplemented with 1 mM ATP and 2 mM MgCl<sub>2</sub>. After 5 min incubation at room temperature, purified DNA-PK (Promega) was added and the reaction mixtures were incubated for 30 min at room temperature after which beads were collected with the magnet without any centrifugation step. The 40 µl supernatant fractions were analyzed by electromobility shift assay by electrophoresis in 0.8% agarose gel in Tris-borate buffer. The beads were washed two times with one volume of binding buffer and finally resuspended in 40 µl of binding buffer without BSA. Proteinase K (40 µg) and Sarkosyl (0.5% final) were added to the bead suspension, incubated for 30 min at 50°C and resolved by electrophoresis in 0.8% agarose gel in Tris-borate buffer.

#### Assessment of XRCC4 phosphorylation by DNA-PK

XRCC4, XLF, and the BRCT domains of DNA Ligase IV were expressed in bacteria and isolated via Ni<sup>2+</sup> agarose affinity as described previously (21). DNA-PK was purchased from Promega (Madison, WI, USA). Proteins (DNA-PK 20 U, XRCC4 2 µg, XLF 2 µg, BRCT 1 µg) were incubated for 60 min at room temperature in kinase active conditions [50 mM HEPES (pH 7.5); 100 mM KCl; 10 mM MgCl<sub>2</sub>; 0.2 mM EGTA; 0.1 mM EDTA; 1 mM DTT; 20 µg/ml calf thymus DNA; 0.1 mM ATP]. Reactions were stopped by the addition of SDS-loading buffer and analyzed either by phosphorimaging or immunoblotting.

## RESULTS

#### XRCC4 mutants that do not interact with XLF restore signal, but not coding end joining in XRCC4-deficient cells

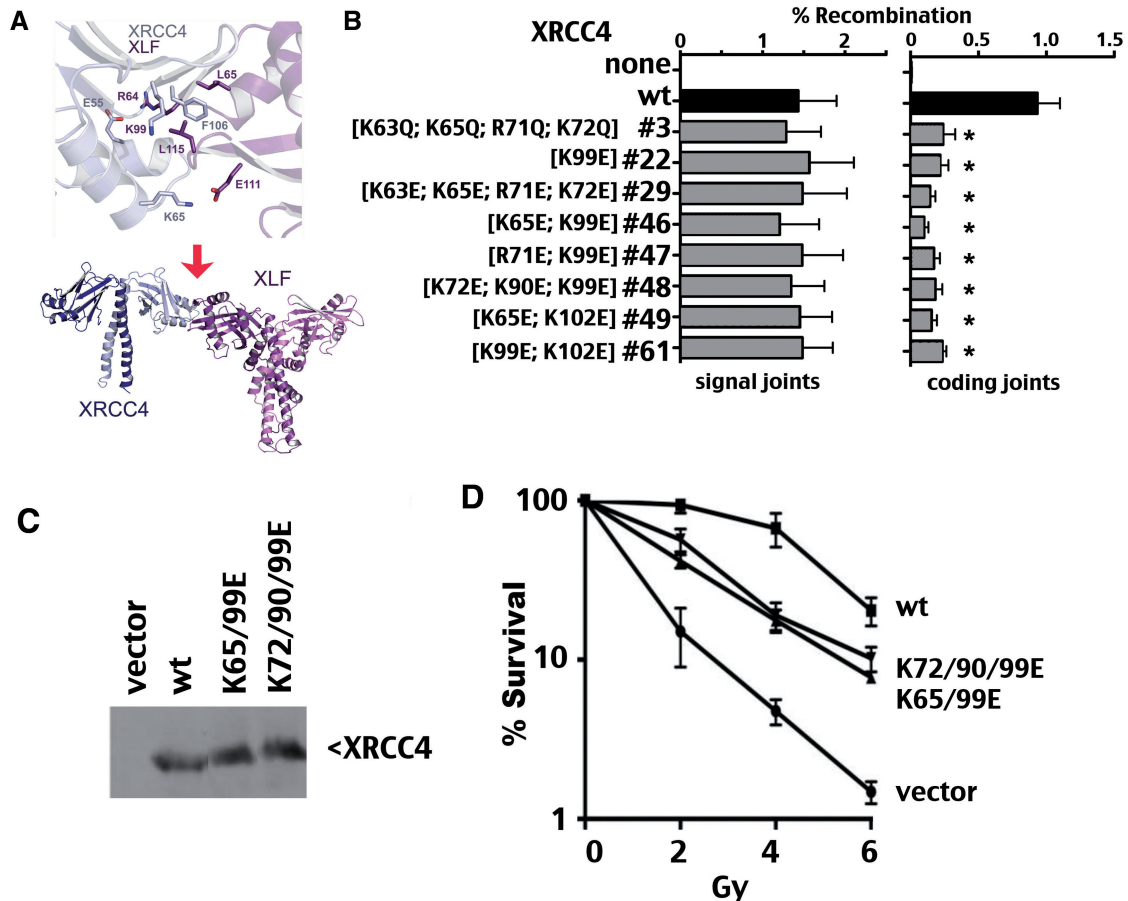
To characterize how XRCC4 and XLF interact, we previously mutated highly conserved XRCC4 residues not predicted to be involved in either dimerization or in DNA Ligase IV interaction (21). Of 17 point or combined XRCC4 mutants, 7 displayed a similar defect in their ability to interact with XLF implicating a cluster of



residues (K63, K65 and K99) at the base of the head as being important for XRCC4's interaction with XLF. Mutation of these residues resulted in XRCC4 molecules that preserved full DNA Ligase IV binding, but abolished interaction with XLF.

To assess the relevance of XRCC4's interaction with XLF in living cells, eight XRCC4 mutants that interact with DNA Ligase IV analogous to wild-type XRCC4, but are markedly defective in their ability to interact with XLF (21), were tested for their ability to support VDJ recombination in XRCC4 deficient CHO cells (XR-1). Previous studies place these residues in the head region of XRCC4 (Figure 1A); the protein-protein interface (top panel, Figure 1A) has been determined (33–37). A useful method to study VDJ recombination is to assess recombination of plasmid substrates introduced into cultured cells ('the Gellert assay') (41). Whereas wild-type XRCC4 and all eight mutants restore similar levels of recombination when assessing signal joints (left panel), only minimal levels of coding joints (right panel) are supported by any of these eight mutants (Figure 1B).

Although all eight mutants display clear deficits in the level of coding end resolution, both support a low level of coding end joining, perhaps analogous to 'leaky' joining observed in cells deficient in Artemis or DNA-PKcs and to a much lower extent in cells deficient in either component of the Ku heterodimer, XRCC4, or DNA Ligase IV. Well-known characteristics of 'leaky' joining, mediated by the a-NHEJ pathway include excessive nucleotide loss, long P elements or extensive use of short sequence homologies. In contrast, successful coding and signal joints formed in the absence of XLF are indistinguishable from those formed in wild-type cells (42,43). To address whether joining mediated by the XRCC4 mutants is analogous to leaky SCID joining, coding joints were sequenced; coding joints mediated by XRCC4 mutants 46 and 48 are indistinguishable from those mediated by wild-type XRCC4 (Table 1). Similarly, signal joints isolated from VDJ assays in cells expressing mutants 46 and 48 displayed 100% fidelity and 95% fidelity respectively, unlike those isolated from cells completely deficient in XRCC4, DNA-PKcs or other c-NHEJ components. These data imply that although the coding end



**Figure 1.** XRCC4 mutants that do not interact with XLF partially restore c-NHEJ deficits in XRCC4 deficient cells. (A) Depiction of the region harboring mutations in XRCC4 that result in disruption of its ability to interact with XLF. (B) Percentage recombination of signal joint substrate (pJH201, left panel) or coding joint substrate (pJH290, right panel) in XR-1 cells transiently expressing wild-type RAGs and wild-type, mutant (46,48), or no XRCC4. Error bars indicate SEM. (C) Immunoblot analysis (25 µg WCE/lane) of XR-1 transfectants expressing wild-type (wt) or mutant (46,48) XRCC4 using a polyclonal rabbit anti-human XRCC4 primary antibody. (D) Radioresistance of XR-1 cells expressing wild-type (wt), mutant (46,48), or no (vector) XRCC4. Error bars indicate SEM.

**Table 1.** Normal end processing of coding and signal joints mediated by XRCC4 mutants that do not interact with XLF

	Coding joints			Signal joints	
	No. of joints sequenced	Nucleotide loss/joint	Percent utilizing SSH	No. of Joints Sequenced	Percent perfect
Wild-type XRCC4	46	3.7	45	40	100
Mutant 46 K65E, K99E	35	4.0	45	38	100
Mutant 48 K72E, K90E, K99E	46	4.7	32	39	94.9

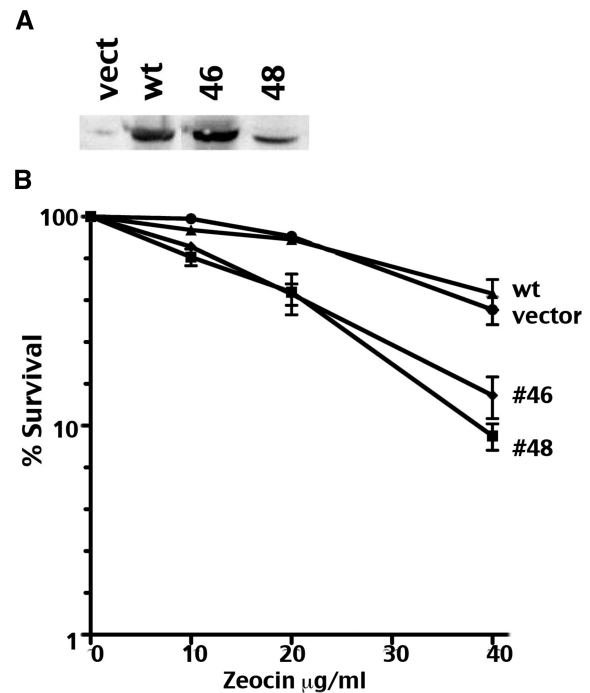
joining rate is diminished, successful joining mediated by XRCC4 mutants 46 and 48 likely proceed by a c-NHEJ mechanism, similar to joints formed in the absence of XLF (42,43).

#### XRCC4 mutants that do not interact with XLF only partially restore radioresistance in XRCC4 deficient cells

We next stably expressed wild-type and mutant XRCC4 in the XR-1 cell strain (Figure 1C), and assessed radiosensitivity. Whereas wild-type human XRCC4 substantially reverses radiosensitivity of XR-1 cells (Figure 1D), both XRCC4 mutants only partially reversed radiosensitivity. We conclude that XRCC4's ability to interact with XLF is functionally important for its role in repairing ionizing radiation induced DNA damage. Moreover, the more pronounced effect on coding (versus signal) end joining observed is somewhat reminiscent of the c-NHEJ phenotypes observed in ATM or MRN deficient cells that have been tentatively attributed to inefficient end synapsis (13–15). These similarities to the ATM/MRN phenotypes coupled with emerging structural data of a stable XRCC4/XLF filament (33–37) suggest a previously unidentified function of XRCC4/XLF complexes in promoting DNA end synapsis.

#### Over expression of XRCC4 mutants that do not interact with XLF radiosensitizes wild-type CHO cells

Data presented thus far and emerging structural data (33,35–37) are consistent with a model whereby XLF/XRCC4 complexes promote synapsis or stability of DNA ends in the repair complex. However, it seems unlikely that this DNA bridging function is strictly required for the c-NHEJ reaction since signal end joining does not require the XRCC4/XLF interaction. It follows that XRCC4/DNA Ligase IV complexes are capable of functioning (without XLF) in the c-NHEJ reaction. To address whether the XRCC4/DNA Ligase IV complex normally functions independently of XRCC4/XLF complexes, or alternatively if DNA Ligase IV is included in XRCC4/XLF complexes (the DNA Ligase IV and XLF interaction regions in XRCC4 are distinct), we tested whether XRCC4 mutants that cannot interact with XLF could exert a dominant negative effect in wild-type cells. If XRCC4/XLF complexes and XRCC4/DNA Ligase IV complexes act independently, over-expression of the mutants (which should sequester endogenous DNA Ligase IV) should not interfere with endogenous XRCC4/XLF complexes. However, if



**Figure 2.** Over-expression of XRCC4 mutants that do not interact with XLF radiosensitizes wild-type CHO cells. (A) Immunoblot analysis (30 µg WCE/lane) of AA8 cells stably expressing wild-type or mutant XRCC4 using a polyclonal rabbit anti-human XRCC4 primary antibody. As can be seen, endogenous hamster XRCC4 is detected in the vector only transfectant (B) Zeocin resistance of AA8 cells over-expressing wild-type or mutant XRCC4 as indicated. Error bars indicate SEM.

DNA Ligase IV functions as part of a potential XRCC4/XLF DNA 'bridging' complex, over-expression of the XRCC4 mutants (and sequestration of all endogenous DNA Ligase IV to mutant XRCC4 complexes) should result in dominant negative inhibition. Over-expression of XRCC4 mutants 46 and 48, but not wild-type human XRCC4 modestly sensitizes the wild-type CHO cell line AA8 to the radiomimetic drug zeocin (Figure 2). This is not a complete block in c-NHEJ activity because neither coding nor signal end joining are significantly inhibited by coexpression of these XRCC4 mutants (data not shown). Although other explanations are possible, these data suggest a functional requirement for DNA Ligase IV occupancy in XRCC4/XLF complexes.

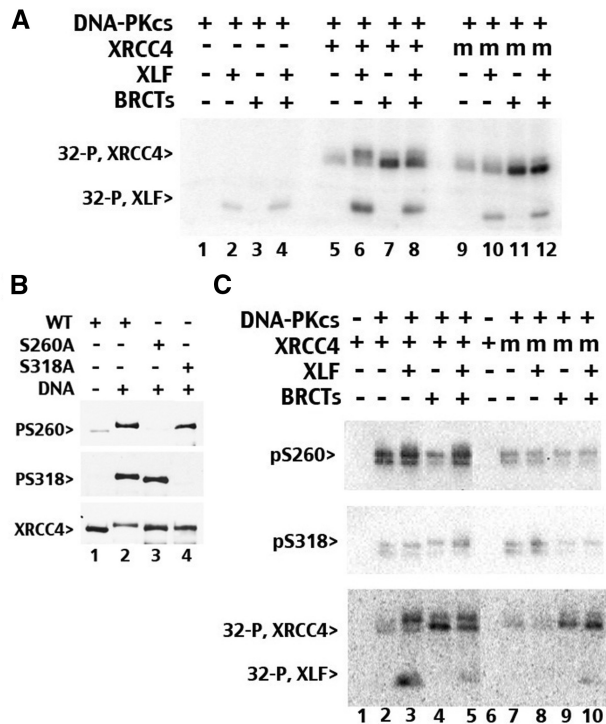
**XRCC4 can be hyper-phosphorylated when in complex with XLF but not when bound to DNA Ligase IV**

XRCC4 and XLF are both robustly targeted by DNA-PK's enzymatic activity *in vitro* and *in vivo* (39,44). Although no functional relevance has been ascribed to XLF or XRCC4 phosphorylations (38,39,44), we considered that different XRCC4 complexes might be targeted by DNA-PK's enzymatic activity differently. To assess phosphorylation of XRCC4/XLF/DNA Ligase IV complexes by DNA-PK, wild-type and mutant XRCC4 as well as XLF and a fragment of DNA Ligase IV encoding the two tandem BRCT domains (BRCTs) were purified from bacteria and used as substrates in kinase assays.

Wild-type XRCC4 (but not mutant 46) promotes phosphorylation of XLF, and XLF promotes phosphorylation of wild-type XRCC4 (but not mutant 46) (Figure 3A, compare lanes 2, 5, 6, 9 and 10). The enhanced phosphorylation of XRCC4 in the presence of XLF is appreciated not only as increased incorporation of phosphorous-32, but also as marked decrease in mobility of a fraction

of the phosphorylated XRCC4, representing hyper-phosphorylated XRCC4 (lane 6). XRCC4 can be phosphorylated on multiple sites, with S260 and S318 being the most prominent sites; S193, S302, S313, T321, S325 and S326 can also be phosphorylated (38). As expected, the effect of enhanced XRCC4 and XLF phosphorylation is not observed with XRCC4 mutant 46 since it does not interact with XLF (compare lanes 5, 6, 9 and 10). In contrast, although the addition of BRCTs clearly enhances phosphorylation of XRCC4, the phosphorylations seem to be less complex because the change in electrophoretic mobility is strongly suppressed (lane 7). As expected, the BRCT domain fragment has a similar effect on phosphorylation of mutant 46 since this mutant interacts with DNA Ligase IV as well as wild-type XRCC4 (compare lanes 9 and 11).

Two phospho-specific reagents (anti-pS260 and anti-pS318) were developed. Validation of the specificity of these reagents was verified (Figure 3B) using S260A and S318A XRCC4 mutants described previously (38). These reagents were used to assess the effect of XLF and BRCT domains on XRCC4 phosphorylation by DNA-PK *in vitro*. Whereas phosphorylation of S260 is enhanced in wild-type XRCC4 by XLF, this is not observed in mutant 46 (Figure 3C). Moreover, the BRCT fragment of DNA Ligase IV inhibits S260 phosphorylation. In contrast, although S318 is clearly phosphorylated by DNA-PK (no S318 phosphorylation is detected in the absence of DNA-PK, Figure 3C, lane 1), neither XLF nor the BRCT fragment of DNA Ligase IV dramatically affects S318 phosphorylation. We conclude that this site is targeted efficiently by DNA-PK whether uncomplexed or complexed with either XLF or the BRCT domain of ligase IV. We conclude that XLF promotes phosphorylation of XRCC4 on S260 and likely other sites as well. In contrast, BRCTs promotes XRCC4 phosphorylation, but not at either S318 or S260. Moreover, BRCTs inhibit XRCC4's hyper-phosphorylation. These data demonstrate that phosphorylation of XRCC4 by DNA-PK varies depending on whether XRCC4 is complexed with XLF or BRCTs.

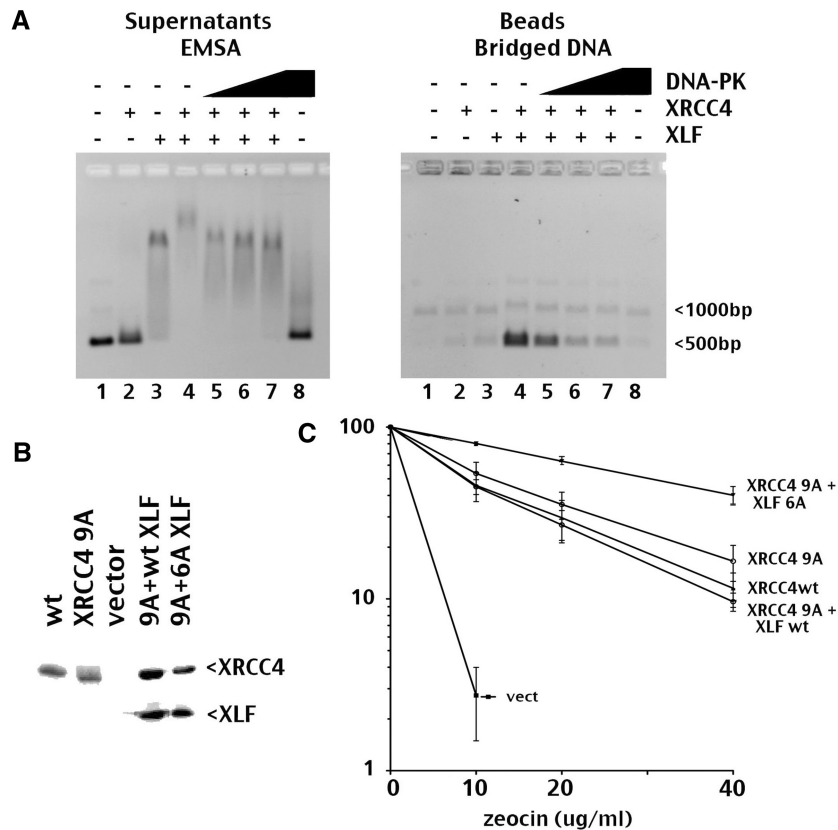


**Figure 3.** XRCC4 can be hyper-phosphorylated when in complex with XLF but not when bound to DNA Ligase IV. (A) Phosphorimaging of SDS-PAGE analyses of *in vitro* kinase assays including DNA-PK, wild-type or mutant XRCC4, wild-type XLF, and the BRCT domains of DNA Ligase IV (BRCTs). (B) Purified wt, S260A or S318A XRCC4 (1 µg) was incubated with DNA-PK under standard assay conditions either in the absence (lane 1) or presence of 10 µg/ml sonicated calf thymus DNA. Reactions analyzed by immunoblotting with phosphospecific antibodies to serine 260 or serine 318, or total XRCC4. (C) Immunoblotting analyses (top panel and middle panel) of *in vitro* kinase assays including DNA-PK, wild-type or mutant XRCC4, wild-type XLF, and the BRCT domains of DNA Ligase IV using phospho-specific antibodies that recognize XRCC4 pS260 (top panel) or XRCC4 pS318 (middle panel). Bottom panel is phosphor image of immunoblot to detect P-32 incorporation in experiment presented in top and middle panels.

**DNA-PK phosphorylation disrupts DNA bridging *in vitro***

In the accompanying manuscript, data is presented demonstrating that XRCC4/XLF filaments bridge DNA molecules *in vitro*. Briefly, an assay was developed to assess bridging of two DNA molecules by recombinant proteins. In this assay, one DNA is biotin labeled and can be immobilized onto streptavidin beads. If bridging occurs (by protein-DNA interaction), the second DNA (different size) will be 'pulled down' onto the streptavidin beads. DNA-protein interactions can be visualized concurrently by electrophoresis of the supernatant fraction. In Figure 4A, the effect of DNA-PK phosphorylation on XRCC4/XLF's filaments ability to bridge DNA was assessed. Consistent with data presented by Andres *et al.* (35), DNA bound XRCC4/XLF multimers are observed by EMSA (left panel, lane 4) and the DNA 'bridged' by XLF/XRCC4 multimers is recovered on beads (right panel, lane 4). However, in the presence of DNA-PK,





**Figure 4.** DNA-PK phosphorylation of the many sites in the C terminal tails of XLF and XRCC4 are functionally redundant and facilitate filament dissociation. (A) Proteins were incubated with streptavidin-coated magnetic beads linked to a biotinylated 1000 bp DNA and free 500 bp DNA. Beads were separated from supernatant and analyzed separately for presence of the 500 bp DNA. An amount of 200 ng each of 1000 and 500 bp DNA fragments were incubated with XRCC4 (2  $\mu$ M) and/or XLF (2  $\mu$ M) and 0, 111, 222 or 333 units of DNA-PK in lanes 4 to 8, respectively. Left panel shows protein–DNA complexes in the supernatants by EMSA. Right panel shows the recovery of DNA species on the beads. (B) Immunoblot analysis (25  $\mu$ g WCE/lane) of XR-1 transfectants expressing wild-type (wt), vector (vect), or 9X ala mutant V5 tagged murine XRCC4 (9A), or the 9Xala mutant in addition to wild-type XLF (wt) or 6X ala mutant human XLF (6A). A monoclonal V5 primary antibody was used to detect murine XRCC4; a polyclonal rabbit anti-human XLF reagent was used to detect XLF. (C) Radioresistance of XR-1 cells expressing wild-type (wt), vector (vect), or 9X ala mutant XRCC4 (9A), or the 9Xala mutant in addition to wild-type XLF (wt) or 6X ala mutant XLF (6A). Error bars indicate SEM.

only the XLF/DNA complex is apparent by EMSA, and we conclude that DNA-PK phosphorylations result in dissociation of XRCC4 from XLF bound DNA. Moreover, DNA bridging is similarly disrupted (right panel, lanes 5–7) reinforcing conclusions of Andres *et al.* (35) that DNA bridging is accomplished by DNA-bound XLF/XRCC4 multimers. In sum, these data suggest that XRCC4/XLF multimer stability and DNA bridging are disrupted by DNA-PK phosphorylation.

**DNA-PK phosphorylation of the many sites in the C-terminal tails of XLF and XRCC4 are functionally redundant; blocking XRCC4/XLF phosphorylation results in increased cell survival after DNA damage**

Considerable effort from our laboratories has focused on determining the functional relevance of DNA-PK targets, including phosphorylations of both XRCC4 and XLF (38,39). Previous mutational analysis of six sites in XLF and nine sites in XRCC4 suggest that these phosphorylations are not critical for c-NHEJ. Phosphorylations of both molecules occur in living cells in the C-terminal

flexible tail domains (not present in crystallographic structures). Modeling and mutational studies suggest that the C-termini of both XRCC4 and XLF may be important for both filament stability and its interaction with DNA (35,36). We considered that the phosphorylation-induced multimer disruption observed *in vitro* (Figure 4A) might be accomplished by phosphorylating the C-termini of either XRCC4 or XLF. To test this possibility, expression constructs encoding wild-type or a mutant XLF with six alanine substitutions of sites in the XLF C-terminus were stably transfected into an XR-1 transfectant that expressed murine XRCC4 with nine alanine substitutions of XRCC4's conserved phosphorylation sites. Over-expression of the 6A XLF mutant markedly protects the 9A XRCC4 transfectant from zeocin induced DNA damage (Figure 4C); this is not just a consequence of XLF over-expression since wild-type XLF expressed in the same cell strain at similar levels does not significantly alter zeocin resistance in XR-1 cells expressing the 9A mutant. We conclude that phosphorylations of XRCC4 and XLF are functionally relevant, but

functionally redundant. Moreover, blocking phosphorylation promotes cell survival after zeocin-induced DNA damage

## DISCUSSION

### VDJ coding end resolution is highly dependent on XRCC4/XLF filaments

A wide variety of XRCC4 mutants have been studied previously, and in all cases mutants that fail to complement the radiosensitivity of XRCC4-deficient cells also have deficits in both signal and coding end joining. Although the observation of an XRCC4 mutation that results in an asymmetric loss in VDJ intermediate resolution is unexpected, asymmetric loss of coding (but not signal) end joining is well appreciated for two other c-NHEJ factors (Artemis and DNA-PKcs) as well as in an emerging group of non-'core' c-NHEJ factors (ATM, MRN, 53BP1) that appear to facilitate coding end synapsis during c-NHEJ (13–15). The asymmetry in coding and signal end resolution in cells lacking Artemis is readily explained by its unique role in opening the hair-pinned termini of cleaved coding ends. The dependence of DNA-PKcs on signal end joining is also variable. Since the assembled DNA-PK complex is fully capable of synapsing DNA ends, it seems possible that DNA-PKcs may also participate in end synapsis, and that variability in signal end joining in different DNA-PKcs deficient cells may be explained by compensation (or lack of compensation) by other factors that can facilitate synapsis. A prevailing consensus has emerged regarding the non-essential role of non-core factors (ATM, MRN, 53BP1); recent reports suggest that these factors facilitate DNA end bridging during c-NHEJ. Thus, defects in these factors impact coding end joining (but not signal end joining) and long-range VDJ joins are more severely affected than proximal VDJ joints. Moreover, Alt and colleagues (45) have demonstrated functional redundancy between either ATM or H2AX and XLF, and have thus added XLF to the list of factors that may facilitate DNA end bridging during c-NHEJ. It is only using the separation-of-function mutants described here that XRCC4's function as a bridging factor (in complex with XLF) is revealed, since XRCC4's function as DNA Ligase IV's cellular cofactor is essential for c-NHEJ.

### Phosphorylation of XRCC4 and XLF is functionally redundant and disrupts XRCC4/XLF filaments

Considerable effort has focused on defining relevant targets of DNA-PK's robust catalytic activity; numerous studies have defined numerous *in vitro* and *in vivo* DNA-PK target sites. Until this study, autophosphorylations within DNA-PKcs were the only phosphorylations shown to be unequivocally relevant for c-NHEJ [reviewed in (46)]. Here, we demonstrate that phosphorylations of the C-termini of XRCC4 and XLF are functional relevant but functionally redundant. Although blocking either XRCC4 or XLF phosphorylation does not affect resistance to ionizing radiation or radio-mimetic drugs, blocking phosphorylation of both XLF and XRCC4

results in enhanced cell survival after zeocin-induced DNA damage. These data coupled with *in vitro* analysis of DNA-PK's effect on XRCC4/XLF complexes suggest that blocking XRCC4 and XLF phosphorylation stabilizes XRCC4/XLF filaments, promoting repair by c-NHEJ. Although other explanations are possible, we hypothesize that DNA-PK phosphorylation of XRCC4/XLF complexes or filaments might function to promote either transition to a ligase active complex or to promote complex dissociation.

Redundancy is an emerging theme for DNA-PK targets. Autophosphorylation of DNA-PKcs occurs on many sites, is functionally complex, and functionally redundant. For several different (phosphorylation modulated) functions, different sites within DNA-PKcs can be phosphorylated to promote the same function (46). The importance of phosphorylation site redundancy in DSB repair is underscored by recent work showing that progressive ablation of 28 distinct ATM/ATR/DNA-PK targets in 53BP1 progressively disrupts function (47).

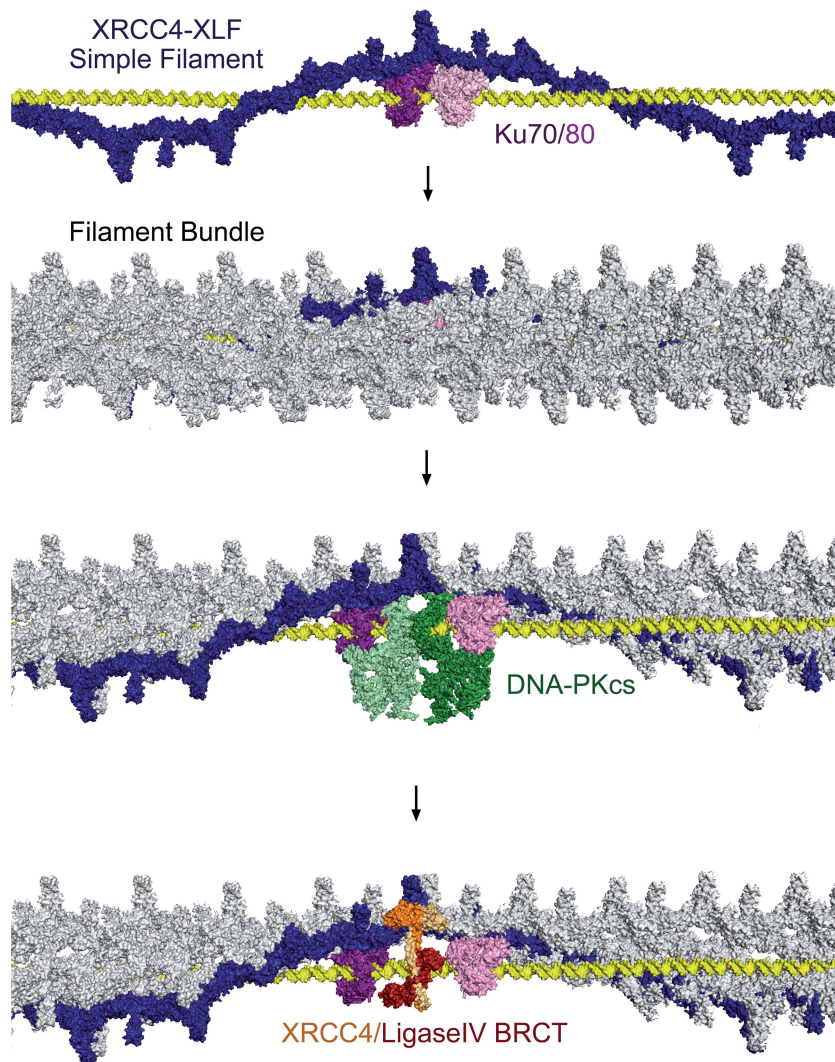
### Do XRCC4/XLF/DNA Ligase IV complexes function as part of larger XRCC4/XLF filaments?

Recent structural studies of XRCC4/XLF filaments (33,35–37) provide the molecular basis for earlier observations of higher order XRCC4 and XLF multimers (22,48). It is intriguing to speculate that these filaments span DNA ends and that perhaps the XRCC4/DNA Ligase IV complex only associates with XRCC4-XLF filaments at the site of damage. Although other interpretations are possible, the fact that XRCC4 mutants that cannot interact with XLF exert a modest dominant negative effect in wild-type cells is consistent with the conclusion that DNA Ligase IV is targeted to DNA ends through its interaction with XRCC4, which is itself part of a larger XRCC4/XLF DNA 'bridging' complex that functions to provide stabilization of the DNA termini during repair.

### An early role for XRCC4/XLF in c-NHEJ

From the findings presented here ascribing XRCC4/XLF complexes to an early step in c-NHEJ, a new model for c-NHEJ emerges (Figure 5). Chen and colleagues (49–51) have shown that XLF is directed to sites of DNA damage by its interaction with Ku (pink/maroon); thus, XRCC4/XLF filaments (dark blue) may be targeted to DSBs via DNA-bound-Ku, functioning to keep broken DNA ends in the same proximity. A single XRCC4/XLF filament bundle [as modeled by Andres *et al.* (35)] is shown in grey (second panel); in their studies multiple filament bundles facilitate parallel bridging of DNA molecules (not illustrated here). The ability of XRCC4/XLF to nucleate into these higher order filaments could not only be important in bridging two DNA ends at a single DSB, but could be a particularly relevant mechanism to facilitate CSR and VDJ. It has been shown that chromosomal regions targeted by RAG or AID for DSBs are repositioned and juxtaposed to defined nuclear domains prior to cleavage by their respective targeting enzymes (52,53). It is intriguing to speculate that recombination (as opposed to rejoining of each individual break) between





**Figure 5.** A model of c-NHEJ. A space-filling model of c-NHEJ complex is presented. DNA is colored in yellow. XRCC4/XLF filaments are shown in dark blue, XRCC4/XLF bundles are shown in grey. DNA-PKcs is colored light and dark green. Ku is colored pink and maroon. The BRCT domains of DNA Ligase IV are shown in red, and the XRCC4 dimer associated with BRCTs is orange.

these paired DSBs might be facilitated by XRCC4/XLF DNA 'bridging' complexes that nucleate at each single DSB that were physically in close proximity because of nuclear repositioning prior cleavage.

We cannot address whether DNA-PKcs (green) or XRCC4/XLF filaments localize to Ku-bound DNA first. However, cellular trafficking and chromatin fractionation experiments suggest that both XRCC4/XLF and DNA-PKcs are independently targeted to Ku-bound DNA although the DNA-PK holoenzyme stabilizes XRCC4's presence at sites of damage (50,49,54).

Implicating XRCC4/XLF complexes in an early stage of c-NHEJ provides a mechanistic explanation for data from Chu and colleagues who used an *in vitro* end joining assay to demonstrate that end processing requires XRCC4 (55). Of course, DNA-PK is also required for regulation of end processing which it mediates by its own autophosphorylation (9,56,57,58).

Here, we show that DNA-PK phosphorylation of the C-terminal tails of XRCC4 or XLF disrupts filament stability and the ability to bridge DNA. We have depicted this event as simplification of the XRCC4/XLF bundle over the break to a single filament (third panel) allowing access to DNA-PKcs (green), and then associated with XRCC4/DNA Ligase IV (red, only BRCT domains depicted, bottom panel). It is also possible that this transition results in a simpler complex of XRCC4/XLF/DNA Ligase IV (not associated with the filament) over the break. Blocking phosphorylation of the XRCC4/XLF filament radio-protects cells, perhaps by stabilizing the XRCC4/XLF filament bundle and allowing c-NHEJ more time to complete repair. These data suggest that DNA-PK phosphorylation of XRCC4/XLF may be important either for transition from complexes that facilitate bridging to complexes that mediate ligation or potentially for complex dissociation.

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