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DNA-PK and ATM phosphorylation sites in XLF/Cernunnos are not required for repair of DNA double strand breaks

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Abstract

Nonhomologous end joining (NHEJ) is the major pathway for the repair of DNA double strand breaks (DSBs) in human cells. NHEJ requires the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Ku70, Ku80, XRCC4, DNA ligase IV and Artemis, as well as DNA polymerases μ and λ and polynucleotide kinase. Recent studies have identified an additional participant, XLF, for XRCC4-like factor (also called Cernunnos), which interacts with the XRCC4-DNA ligase IV complex and stimulates its activity *in vitro*, however, its precise role in the DNA damage response is not fully understood. Since the protein kinase activity of DNA-PKcs is required for NHEJ, we asked whether XLF might be a physiological target of DNA-PK. Here, we have identified two major *in vitro* DNA-PK phosphorylation sites in the C-terminal region of XLF, serines 245 and 251. We show that these represent the major phosphorylation sites in XLF *in vivo* and that serine 245 is phosphorylated *in vivo* by DNA-PK, while serine 251 is phosphorylated by Ataxia-Telangiectasia Mutated (ATM). However, phosphorylation of XLF did not have a significant effect on the ability of XLF to interact with DNA *in vitro* or its recruitment to laser-induced DSBs *in vivo*. Similarly, XLF in which the identified *in vivo* phosphorylation sites were mutated to alanine was able to complement the DSB repair defect as well as radiation sensitivity in XLF-deficient 2BN cells. We conclude that phosphorylation of XLF at these sites does not play a major role in the repair of IR-induced DSBs *in vivo*.

Keywords

XLF; DNA-PK; Phosphorylation; Nonhomologous end joining

1. Introduction

In human cells, DNA double strand breaks (DSBs) are repaired by either the homologous recombination pathway or by non-homologous end joining (NHEJ). Proteins required for NHEJ include the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), the

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Appendix A. Supplementary data: Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2008.06.015.

Ku70/80 heterodimer and the XRCC4-DNA ligase IV complex. Absence or inactivation of any of these factors leads to radiation sensitivity and defects in DNA DSB repair and V(D)J recombination [1–3]. Additional proteins such as Artemis, polynucleotide kinase (PNK) and DNA polymerases of the Pol X family are also required for NHEJ under some circumstances (reviewed in [4]). In 2006, another factor, named XLF for XRCC4-like factor (also called Cernunnos), was identified. XLF interacts with XRCC4 and cells that lack XLF are radiation sensitive and defective in DSB repair and V(D)J recombination [5–7]. XLF is similar in structure to XRCC4 in that the first ~233 amino acids adopt a globular head domain that is followed by a long, α -helical stalk [5,8,9]. However, unlike XRCC4, the C-terminal stalk in XLF doubles back on itself to form a cone-shaped structure [8,9]. The structure of the C-terminal ~50 amino acids of XLF is not known, although it has been predicted to facilitate DNA binding *in vitro* [8]. Several recent studies have shown that XLF stimulates the activity of the XRCC4-DNA ligase IV complex *in vitro* [10–12]. However, its precise role in NHEJ remains to be determined.

One of the important players in NHEJ is DNA-PKcs. DNA-PKcs is a member of the phosphatidylinositol-3 kinase-like (PIKK) family of protein kinases which includes Ataxia-Telangiectasia Mutated (ATM) and ATM- and Rad3-related (ATR) [13]. Given that the protein kinase activity of DNA-PK is required for NHEJ [14–16], one of the major challenges to understanding its function in DSB repair is to identify its physiologically relevant substrates. Like ATM and ATR, DNA-PKcs phosphorylates its substrates on serines or threonines that are followed by glutamine (*i.e.* SQ/TQ sites), however, it can also phosphorylate substrates on serines and threonines that are followed by other amino acids (reviewed in [3]). *In vitro*, DNA-PK substrates include many of the proteins involved in NHEJ including DNA-PKcs itself, Ku70, Ku80, XRCC4, DNA ligase IV and Artemis (reviewed in [4]). However, the *in vitro* DNA-PK phosphorylation sites in Ku70, Ku80 and XRCC4 are not required for NHEJ [17–19]. DNA-PKcs is also autophosphorylated *in vitro* and is phosphorylated on multiple sites *in vivo*. Phosphorylation of DNA-PKcs likely regulates access of other repair factors to DSBs and is required for NHEJ (reviewed in [4,20]). XLF, the newest member of the NHEJ pathway, contains several SQ/TQ motifs (S55, S132, T223 and S251), and therefore we considered that it might be a substrate for DNA-PK.

Here, we show that XLF is phosphorylated *in vitro* by DNA-PK on multiple sites including serines 245 and 251 and that these sites are phosphorylated *in vivo* in irradiated human cells. Using phosphospecific antibodies, we show that IR-induced phosphorylation of serine 245 is largely DNA-PK-dependent, while IR-induced phosphorylation of serine 251 is ATM-dependent. The major phosphorylation sites are located in the extreme C-terminal region of XLF, which we show directly binds DNA *in vitro*. However, phosphorylation by DNA-PK did not have a major effect on the ability of XLF to interact with DNA. XLF has recently been shown to be recruited to sites of UV laser-induced DNA damage *in vivo* [21]. We show that XLF in which six of the identified *in vivo* phosphorylation sites have been mutated to alanine (6A-XLF) is still recruited to sites of UV laser-damaged DNA. Moreover, 6A-XLF is still able to complement the repair defect in XLF-deficient 2BN cells [22,23] with respect to kinetics of histone H2AX phosphorylation as well as survival after IR. Therefore, we conclude that although XLF is phosphorylated in a PIKK-dependent manner *in vivo*, phosphorylation at these sites is unlikely to be required for repair of IR-induced DSBs *in vivo*.

2. Materials and methods

2.1. Cloning and expression of human XLF in bacteria

Full-length human XLF (hXLF) cDNA (Genbank accession number: NM 024782) was cloned from a human cDNA library as described previously [18]. The sequences of the primers used (GST-hXLF) are provided in Supplementary methods.

The PCR product was subcloned into the pGEX6P1 vector (GE Healthcare) at BamHI/XmaI sites to create a GST-fusion protein, or into pQE30 (QIAGEN) vector at BamHI/XmaI sites for a His-tagged protein, respectively. DNA sequences were confirmed by the University of Calgary DNA Services Facility. The pGEX6P1-XLF plasmid was introduced into *Escherichia coli* BL21 for protein expression and GST-tagged XLF protein was purified over glutathione-conjugated sepharose (GE Healthcare). The pQE30-XLF plasmid was introduced in *E. coli* M15 for expression and the His-XLF protein was purified over Ni-NTA agarose (Qiagen). The conditions used for expression and purification of GST- and His-tagged proteins in bacteria are provided in Supplementary methods.

2.2. Mutational analysis of XLF

N- and C-terminal deletions of XLF were generated by PCR using oligonucleotide 5'-GST-hXLF and the primer 3'-hA239, or oligonucleotide 3'-GST-hXLF and primer 5'-hΔ240, respectively (see Supplementary methods for details). These constructs were expressed in bacteria to generate a C-terminal deletion mutant corresponding to amino acids 1–239 (GST-XLF_{1–239}) and an N-terminal deletion mutant corresponding to amino acids 240–299 (GST-XLF_{240–299}). Conditions for the expression and purification of XLF are described in Supplementary methods. Site direct mutagenesis was used to generate single serine or threonine to alanine mutations of hXLF at serines 55, 132, 203, 245, 251 and 263 and threonines 223 and 266. PCR was performed for each designed mutation with the following sets of complementary primers: 5'-h55S>A, 3'-h55S>A, 5'-h132S>A, 3'-h132S>A, 5'-h203S>A, 3'-h203S>A, 5'-h223T>A, 3'-h223T>A, 5'-h245S>A, 3'-h245S>A, 5'-h251S>A, 3'-h251S>A, 5'-h263S>A, 3'-h263S>A, 5'-h266T>A, 3'-h266T>A (see Supplementary methods for details). The same primers were used to generate mutations in pGEX6P1 (for bacterial expression), and pIRES-hrGFP-1a-hXLF and pEGFP-C2-hXLF for expression in mammalian cells (see below).

2.3. Generation of vectors for transient expression of XLF in human cells

hXLF DNA was amplified using pGEX6P1-XLF as a template and the PCR product was subcloned into either the mammalian expression vector pIRES-hrGFP-1a (Stratagene) to generate XLF containing a C-terminal FLAG-tag or pEGFP-C2 (BD Biosciences) to generate XLF containing an N-terminal GFP fusion. The primers used (5'-hXLF-FLAG, 3'-hXLF-FLAG, 5'-EGFP-hXLF and 3'-EGFP-hXLF) are shown in Supplementary methods. The quadruple phosphorylation mutant (4A; S132/203/245/251A) and the sextuple mutant (6A; S132/203/245/251/S263/T266A) were generated in pIRES-hrGFP-1a and/or pEGFP-C2 using the primers described above.

2.4. Immunoblotting

Samples were subjected to SDS PAGE, transferred to nitrocellulose and probed as described previously [18]. The generation of antibodies used in this study is described in the Supplementary methods section.

2.5. Identification of *in vitro* phosphorylation sites

In vitro phosphorylation assays were carried out exactly as described previously [18]. DNA-PKcs and Ku were purified from HeLa cells as described previously [24]. For small-scale reactions (20 μ l final volume), 1 μ g GST-XLF was used. For preparation of samples for mass spectrometry, reactions contained 20 μ g of GST-XLF bound to beads and were scaled up accordingly. Beads were washed 5 times each with 1ml IP buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM KCl, 1 mM EDTA) containing 0.25% (v/v) Triton X-100, followed by 10 $\times$ 1ml washes of ice-cold phosphate buffered saline (PBS). Samples were analyzed by mass spectrometry by WEMB Biochem. Inc., Toronto, Canada (<http://www.wembbiochem.com/>) as described previously [25]. Briefly, samples were digested with both trypsin and chymotrypsin at 32°C overnight. Samples were then acidified to pH ~2, and phosphopeptides were selected using a Fe(III)-loaded pipette tip and analyzed on an LCQ Deca XP spectrometer (Thermo Finnegan). The data were analyzed using Bioworks 3.1 and peptide sequence data were rechecked manually.

2.6. *In vivo* labeling

Circular plasmid encoding XLF-FLAG DNA was transfected into human HeLa cells using Lipofectamine (Invitrogen) according to the manufacturers recommended conditions. Transfected cells were maintained in DMEM containing 5% fetal calf serum (FCS) (Hyclone) in the absence of antibiotics for 24 h at 37°C under 5% CO₂. Prior to *in vivo* labeling, cells were incubated for 2 h in phosphate-free DMEM (Invitrogen) containing 10% FCS. Protein kinase inhibitors (suspended in DMSO), or an equivalent volume of DMSO, were added directly to the medium as indicated and cells were incubated at 37°C, under 5% CO₂ for 30min. Four hundred microCuries of ³²P inorganic phosphate (10 milliCuries/ml, GE Healthcare) was added to each flask (containing 2 ml of phosphate-free media plus 10% FCS) and cells were returned to the incubator for 30min. Where indicated, cells were irradiated with 10 Gy IR using a ¹³⁷Cs source GammaCell 1000 Tissue Irradiator (MDS Nordion) and incubated at 37°C under 5% CO₂ for an additional 2 h. The radioactive media was removed and the cells were washed five times in ice cold PBS. Cells were lysed by incubation on ice for 10 min in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1 μ M microcystin-LR, 0.2 mM PMSF, 0.2 μ g/ml leupeptin and 0.2 μ g/ml aprotinin. Extracts were centrifuged at 10,000 $\times g$ for 10 min to remove precipitates and protein concentrations were determined using the BioRad Detergent Compatible Protein Stain using BSA as standard. Extracts from radioactively labeled cells were used immediately for immunoprecipitation assays. One mg (total protein) of each sample was added to 30 μ l of 1:1 slurry of FLAG antibody conjugated agarose (Sigma-Aldrich) and samples were incubated for 4h at 4°C with rotation. Samples were then washed five times each with 1 ml of IP buffer containing 0.25% (v/v) Triton X-100, and precipitates were analyzed by autoradiography after SDS PAGE on 10% polyacrylamide gels.

2.7. Identification of *in vivo* phosphorylation sites

XLF-FLAG was immunoprecipitated from either unirradiated or irradiated HeLa cells (grown in the absence of ³²P) as described above. The beads were washed five times each with 1ml of IP buffer containing 0.25% (v/v) Triton X-100, then 10 times with 1 ml per wash ice-cold PBS and analyzed by mass spectrometry at WEMB Biochem. Inc. as described above.

2.8. EMSA assays

Full-length GST-XLF as well as C- and N-truncation mutants (GST-XLF₁₋₂₃₉ and GST-XLF₂₄₀₋₂₉₉) were expressed and purified as described above. DNA fragments of 100,200 and 300 bp were PCR amplified from plasmid DNA, gel purified and end-labeled with ³²P-

γ -ATP using T4 polynucleotide kinase (Invitrogen) according to the manufacturers recommended procedures, then purified using a QIAquick® PCR purification kit (Qiagen). Protein binding was carried out in 25 mM HEPES–NaOH (pH 7.4), 50 mM NaCl, 1mM EDTA, 1mM DTT, 5% (v/v) glycerol plus BSA at 100 μ g/ml or as indicated. Purified GST-tagged XLF, GST alone or BSA was added at the concentrations indicated. Approximately 0.8fmol of radioactively labeled DNA was added and reactions were incubated at room temperature for 30 min. Protein–DNA complexes were resolved by non-denaturing gel electrophoresis on Tris–glycine, 4.8% acrylamide gels as described previously [26]. Where indicated, 0.7 μ g of either purified IgG to phosphorylated serine 251 or control IgG was added to reactions after the addition of DNA. Samples were incubated for 30 min and analyzed by EMSA as above.

2.9. Phosphorylation of XLF-C-terminal fragments for EMSA Assays

GST-XLF_{240–299} was immobilized on GST-beads and phosphorylated by DNA-PK as above, except that reactions contained 800 μ g of GST-XLF_{240–299} and 170 μ g of purified DNA-PK complex. Phosphorylation reactions contained 0.25 mM ATP, while mock phosphorylation contained DNA-PKcs, Ku, DNA but no ATP. After incubation at 30°C for 30 min., beads were washed with 50 mM Tris–HCl, pH 8.0, containing 250 mM NaCl and 0.2 mM PMSF to remove components of the phosphorylation or mock phosphorylation reactions. The GST-tagged protein was then eluted from the beads with 50 mM Tris–HCl, pH 8.0 containing 20 mM glutathione, 2 mM DTT, 0.2 mM PMSF, 0.2 μ g/ml leupeptin and 0.2 μ g/ml pepstatin. GST-XLF_{240–299} containing fractions were concentrated with an Amicon Ultra Centrifugal Filter (30 kDa MW cutoff) and the buffer was changed to 50 mM Tris–HCl, pH 8.0.

2.10. Live cell imaging

U2OS cells were transiently transfected with EGFP-tagged wt-XLF, 4A-XLF, or 6A-XLF exactly as described previously [21]. Forty-eight hours after transfection, DSB induction by laser micro-irradiation and time-lapse imaging were carried out. Fluorescence intensities of undamaged and damaged areas were determined using the Axiovision software (Version 4.5, Carl Zeiss). All measurements were corrected for nonspecific bleaching during monitoring, and relative fluorescence was calculated by the formula described by Uematsu et al. [27]. Mean values of relative fluorescence intensities at each time point were calculated from 10 independent measurements. FRAP analysis was carried out as described previously [21,27] with slight modifications. Cells were micro-irradiated to induce DSBs as described above and incubated for 5min at 37°C to allow maximum accumulation of EGFP-XLF at the damaged sites. FRAP measurement was performed for up to 2min. Fluorescence of the undamaged and laser-damaged nuclear areas at a specific time point after photobleaching ($F_{\text{Nuc}}(t)$ and $F_{\text{LD}}(t)$, respectively) were determined using Axiovision software. Fluorescence recovery (F_{Rec}) was calculated by the following formula: $F_{\text{Rec}}(t) = (F_{\text{LD}}(t)/F_{\text{LD}}(0)) \times (F_{\text{Nuc}}(0)/F_{\text{Nuc}}(t))$, where $F_{\text{LD}}(0)$ and $F_{\text{Nuc}}(0)$ are the fluorescence intensities of the laser-damaged and undamaged nuclear areas just before photobleaching, respectively. The F_{Rec} before micro-irradiation was set to 1.0 and the F_{Rec} after bleaching was set to 0 for the calculation of relative fluorescence.

2.11. H2AX phosphorylation assays

Tert-immortalized XLF-deficient human fibroblasts (2BN cells, [22]) were transfected with pEGFP-C2-vector alone, pEGFP-wt-XLF, or pEGFP-6A-XLF using Nucleofector Kit R (Amaxa) according to the manufacturers recommended conditions. Cells were plated on coverslips and after 24 h, irradiated with 2 Gy, then incubated at 37°C under CO₂ for the times indicated. Cells were fixed in 3.7% (v/v) formaldehyde in 1 $\times$ PBS for 10 min then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in 1 $\times$ PBS for 45 s. Cells were

washed 5×1 min with $1 \times$ PBS. γ -H2AX was detected using a monoclonal antibody to serine-139 phosphorylated H2AX (Upstate 1:1000 dilution in 1% BSA for 1 h). Cells were washed as above and then incubated for 30 min under dark conditions with Alexa-594 conjugated goat anti mouse secondary antibody (Molecular Probes, Eugene, OR) at 1:500 dilution in 1% BSA in $1 \times$ PBS. Cells were washed as above. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich) ($1 \mu\text{g/ml}$ in $1 \times$ PBS) for 5 min. Cells were washed 3×2 min with $1 \times$ PBS and coverslips were mounted onto slides using Vectashield (Vector Laboratories Inc., Burlingame, CA). γ -H2AX foci were visualized on a Leica DMRXA2 microscope as described previously [28]. γ -H2AX foci in GFP-labeled cells were counted by eye by two independent viewers. At least 30 cells were counted for each time point. In the experiments shown, the number of foci in GFP-labeled cells was compared to those in non-GFP-labeled cells on the same coverslip.

2.12. Clonogenic survival assays

2BN cells were transfected with pEGFP-C2-vector alone, pEGFP-wt-XLF, or pEGFP-6A-XLF as describe above. After 24 h, cells were irradiated with 1Gy, seeded and incubated at 37°C under CO_2 . After 14 days, cells were fixed in 3% acetic acid/8% methanol, stained with 0.2% crystal violet in 10% formalin/PBS and colonies were counted as described previously [18]. Each data point is the average of three samples and results are representative of three separate experiments.

3. Results

3.1. DNA-PK phosphorylates XLF *in vitro*

XLF was expressed in *E. coli* and purified as described in Section 2. The purified protein, which ran as a single band at approximately 37kDa on SDS PAGE (Fig. 1A), was phosphorylated by DNA-PK *in vitro* (Fig. 1B). Phosphoamino acid analysis revealed that XLF was phosphorylated predominantly on serine but some phosphothreonine was also detected (data not shown). To determine whether XLF was phosphorylated on SQ/TQ motifs, phosphorylated XLF was analyzed by western blot using a phosphospecific antibody to ATM/ATR substrates (Cell Signaling Inc.). This antibody recognized phosphorylated XLF as well as autophosphorylated DNA-PKs (which is phosphorylated *in vitro* on SQ/TQ sites [29]), but not the Ku70/80 heterodimer which is phosphorylated at non-SQ/TQ sites *in vitro* [30] (Fig. 1C). As expected, phosphorylation of XLF was inhibited by the PIKK inhibitor, wortmannin (Fig. 1C). Thus, DNA-PK phosphorylates XLF *in vitro*, on sites that include SQ/TQ sites.

We next proceeded to identify the *in vitro* DNA-PK phosphorylation sites in XLF using mass spectrometry (MS). GST-XLF was purified on glutathione beads, phosphorylated by purified DNA-PK, digested with trypsin and chymotrypsin and analyzed by MS/MS. Two phosphorylation sites corresponding to serines 245 and 251 were identified (Supplementary Fig. 1A and B). To confirm these assignments and to estimate the relative amount of phosphorylation at each site, serine/threonine to alanine mutations were generated in full-length GST-XLF and proteins were expressed and tested for their ability to be phosphorylated by DNA-PK *in vitro*. Mutation of S245 or S251 to A reduced phosphorylation by 60% and 75%, respectively (Fig. 1D), indicating that these are the major DNA-PK phosphorylation sites. Subsequent MS/MS analysis indicated that serine 263 and threonine 266 were also phosphorylated (Supplementary Fig. 1C and D), however, mutation analysis revealed that these sites contribute to approximately 10% of the total phosphorylation of XLF *in vitro* (data not shown).

To further confirm the results of MS and mutation analysis, N- and C-terminal truncation mutants of XLF were generated and tested for phosphorylation by DNA-PK. Consistent with

the results from single amino acid mutagenesis, a C-terminal fragment of XLF corresponding to amino acids 240–299 (GST-XLF_{240–299}) was highly phosphorylated *in vitro* by DNA-PK, while an N-terminal fragment, corresponding to amino acids 1–239 (GST-XLF_{1–239}) was not (Fig. 1E). We conclude that the major *in vitro* DNA-PK phosphorylation sites in XLF are located in the C-terminal 60 amino acids of XLF (amino acids 240–299) and that serines 245 and 251 are the major phosphorylation sites, while serine 263 and threonine 266 represent minor sites.

3.2. XLF is phosphorylated *in vivo*

To determine whether XLF was phosphorylated *in vivo*, full-length human XLF was cloned into the pIRES-hrGFP-1a vector to create a C-terminal FLAG-tagged fusion protein (XLF-FLAG). XLF-FLAG was transfected into HeLa cells and cells were metabolically labeled with ³²P-inorganic phosphate as described in Section 2. XLF was immunoprecipitated using anti-FLAG conjugated beads and analyzed by SDS PAGE, immunoblot and autoradiography (Fig. 2A). Where indicated, cells were treated with either wortmannin, the specific DNA-PK inhibitor NU7441 [16] or the specific ATM inhibitor KU55933 [31] 30min prior to irradiation. XLF-FLAG was phosphorylated *in vivo* in the absence of IR and irradiation did not increase the amount of phosphate incorporation (Fig. 2A, lanes 2 and 3), suggesting either that XLF is constitutively phosphorylated *in vivo* or that either transfection or the presence of radioisotope during the *in vivo* labeling reaction induced phosphorylation. Significantly, addition of either wortmannin, NU7441 or KU55933, reduced phosphorylation by approximately 60–70% (Fig. 2A, lanes 4–6). In summary, XLF is phosphorylated *in vivo* in a PIKK-dependent manner.

To identify the *in vivo* phosphorylation sites in XLF, HeLa cells were transfected with XLF-FLAG, irradiated with 10Gy IR and XLF was immunoprecipitated, digested and analyzed by MS as described in Section 2. Phosphorylation was detected at the previously identified *in vitro* phosphorylation sites (serines 245 and 251) as well as serines 132, 203 and 263 (Supplementary Fig. 2, panels A–E). Phosphorylation at threonine 266 was also indicated in some experiments (Supplementary Fig. 2, panel F). To confirm these assignments, HeLa cells were then transfected with XLF-FLAG containing S to A mutations at serines 132, 203, 245, or 251 or the quadruple mutant S132/203/245/251A (labeled XLF-4A in Fig. 2B, lane 7) and phosphorylation was determined by *in vivo* labeling. Mutation of S132 had no appreciable effect on the amount of *in vivo* phosphorylation, while mutation of serines 203, 245 or 251 reduced phosphorylation by approximately 35, 65 or 50% respectively (Fig. 2B). Mutation of all four sites reduced phosphorylation by more than 70%, suggesting that serines 203, 245 and 251 are the major *in vivo* phosphorylation sites in XLF. From MS/MS analysis, we speculate that the additional *in vivo* phosphorylation sites include serine 263 and possibly threonine 266 (Supplementary Fig. 2, panels E and F). In summary, XLF is phosphorylated *in vivo* on serines 203, 245 and 251, and these three sites account for approximately 70% of the total *in vivo* phosphorylation of XLF, while serine 263 and threonine 266 may be minor *in vivo* phosphorylation sites.

To further characterize *in vivo* phosphorylation of XLF, phosphospecific antibodies were generated to phosphorylated serines 245 and 251 (see Supplementary materials and methods). The antibodies were affinity purified, tested against DNA-PK-phosphorylated XLF and shown to be specific for the respective phosphorylation sites (Fig. 3). HeLa cells were transfected with either wild type XLF-FLAG, XLF-FLAG with a serine to alanine mutation at serine 245 (XLF-S245A) or FLAG-tagged XLF with a serine to alanine mutation at serine 251 (XLF-S251A). Twenty-four hours post-transfection, cells were irradiated and after 2h, whole cell extracts were generated as described in Section 2. XLF was immunoprecipitated and analyzed using the phosphospecific antibodies to serines 245 and 251. Phosphorylation of serine 245 was detected in transfected, unirradiated cells and

phosphorylation was not increased after IR (Fig. 3A, upper two panels). In contrast, phosphorylation of serine 251 was low in unirradiated transfected cells but was IR-inducible (Fig. 3A, lower two panels). Phosphorylation at serine 251 was detectable within 1h of IR and remained phosphorylated for at least 4h (Fig. 3B).

The different kinetics of phosphorylation at serines 245 and 251 suggested that different protein kinases might be responsible for phosphorylation. Cells were therefore transfected with wild type XLF and, after 24 h, incubated with either the DNA-PK inhibitor NU7441 or the ATM inhibitor KU55933 for 30 min prior to irradiation. After 2h, cells were harvested, and XLF was immunoprecipitated and analyzed for phosphorylation at serines 245 and 251. Pretreatment with the DNA-PK inhibitor NU7441 ablated phosphorylation at serine 245 but had no effect on phosphorylation of serine 251 (Fig. 3C, lanes 3–4), suggesting that DNA-PK phosphorylates serine 245 but not serine 251 *in vivo*. In contrast, pretreatment with the ATM inhibitor KU55933 ablated phosphorylation of serine 251, showing that this site is clearly ATM-dependent *in vivo* (Fig. 3C, lower two panels). We also noted that at higher concentrations, KU55933 resulted in some inhibition of phosphorylation of serine 245 (Fig. 3C, lanes 5–6). This may indicate that ATM also contributes to the phosphorylation of serine 245 *in vivo*, however, it is also possible that when added to cells at 10 μ M, KU55933 may partially inhibit DNA-PK activity, as the IC₅₀ for inhibition of DNA-PK activity *in vitro* is 2.5 μ M [31]. We therefore conclude that phosphorylation of serine 245 is largely DNA-PK-dependent, while phosphorylation of XLF at serine 251 is ATM-dependent *in vivo*.

Since these experiments were carried out on transfected cells, we next wanted to determine whether endogenous XLF was phosphorylated in untransfected cells. Preliminary experiments indicated that the amount of XLF in most human cell lines was relatively low (data not shown), therefore, for these studies, XLF was partially purified from either unirradiated or irradiated HEK293 cells (see Supplementary methods) and analyzed using the phosphospecific antibodies to serines 245 and 251. No phosphorylation of either serine 245 or 251 was detected in unirradiated cells (Fig. 4, lane 1). However, when XLF was purified from irradiated cells, phosphorylation at both sites was detected (Fig. 4, lane 3). Therefore, endogenous XLF is phosphorylated on serines 245 and 251 in irradiated but not unirradiated human cells.

3.3. The C-terminal 60 amino acids of XLF are required for interaction with DNA, but phosphorylation does not have a major effect on DNA binding *in vitro*

Having shown that XLF is phosphorylated at serine 245 and serine 251 *in vivo*, we next attempted to elucidate the function of C-terminal phosphorylation of XLF. It has recently been reported that deletion of ~70 amino acids from the C-terminus of XLF (amino acids 225–299) reduces the ability of XLF to interact with DNA *in vitro* [8]. Since the majority of the identified *in vitro* and *in vivo* phosphorylation sites are located in this C-terminal region, we hypothesized that phosphorylation of XLF might regulate the ability of XLF to interact with DNA. To test this hypothesis, full-length GST-XLF, GST-XLF_{1–239} and GST-XLF_{240–299} were expressed in bacteria and purified to homogeneity. The ability of these fragments to bind DNA was then determined in EMSA assays. As reported previously, the ability of full-length XLF to interact with DNA was length-dependent [12] (Fig. 5A–C, right hand panel, lanes 2–5). Moreover, the C-terminal fragment of XLF (amino acids 240–299) bound significantly more strongly than either full-length XLF, or XLF_{1–239}, providing direct evidence that the majority of the DNA binding activity of XLF resides in the C-terminal region (amino acids 240–299) (Fig. 5B and C, lanes 10–13). As reported previously for full-length XLF, GST-XLF_{240–299} bound more strongly to longer fragments of DNA [12].

We next asked whether phosphorylation by DNA-PK affected the ability of XLF to bind to DNA. The C-terminal fragment of XLF (GST-XLF₂₄₀₋₂₉₉) was either mock phosphorylated (*i.e.* incubated with DNA-PK and DNA but no ATP) (Fig. 6A, lane 2), or phosphorylated (incubated with DNA-PK, DNA and ATP) (Fig. 6A, lane 3), then eluted from the glutathione column. Phosphorylation of GST-XLF₂₄₀₋₂₉₉ was confirmed in the DNA-PK-phosphorylated sample by the presence of a series of supershifted bands on SDS PAGE (Fig. 6A, lane 3), using the phosphospecific antibody to serine 245 (Supplementary Fig. 4) and by phosphorylation in the presence of ³²P- γ -ATP (data not shown). Immunoblotting for DNA-PKcs and Ku confirmed that neither mock phosphorylated nor phosphorylated XLF protein was contaminated with DNA-PK after elution from the beads (Supplementary Fig. 4). Equimolar amounts of non-phosphorylated, mock-phosphorylated and phosphorylated GST-XLF₂₄₀₋₂₉₉ were then assayed for their ability to interact with DNA in EMSAs. The results show that phosphorylation of the C-terminal region of XLF did not have a significant effect on its ability to interact with DNA (Fig. 6B). Because the phosphorylated XLF₂₄₀₋₂₉₉ fragment likely contained a mixture of phosphorylated and non-phosphorylated protein species, it was important to determine whether the phosphorylated form of XLF₂₄₀₋₂₉₉ was actually present in the DNA-protein complex detected on EMSA. To do this, purified IgG from either phosphospecific antibody 251 or control IgG was added to reactions prior to DNA binding. IgG to phosphoserine 251 shifted the protein-DNA complex, whereas control IgG did not (Fig. 6C), confirming that the protein-DNA complexes do indeed contain phosphorylated XLF. From these experiments, we conclude that phosphorylation of the C-terminus of XLF does not have a major effect on its ability to bind DNA.

3.4. The C-terminal phosphorylation sites are not required for localization of XLF to DSBs *in vivo*

Although phosphorylation of XLF did not prevent XLF from binding to DNA *in vitro*, it was important to determine whether phosphorylation affected its ability to interact with DNA *in vivo*. To do this, EGFP-tagged wt-XLF or XLF containing mutations at four (S132, S203, S245 and S251) or six (S132, S203, S245, S251, S263 and T266) of the identified *in vivo* phosphorylation sites (EGFP-wt-XLF, EGFP-4A-XLF, or EGFP-6A-XLF, respectively), was transiently transfected into human osteosarcoma U2OS cells. DNA damage was induced using a 365nm UV laser and the kinetics of recruitment of EGFP-proteins to sites of damage was examined using live cell imaging exactly as described previously [21,27]. Both wild-type and phospho-mutants were found to accumulate at sites of laser-induced DNA damage with similar kinetics (Fig. 7A), therefore phosphorylation at these sites is not required for recruitment to DSBs *in vivo*.

Next, we analyzed XLF turnover at DSBs in the initial accumulation phase by fluorescence recovery after photobleaching (FRAP). A small area in a nucleus was micro-irradiated and cells were incubated for 5min to allow EGFP-XLF accumulation to reach steady-state levels. Subsequently, the damaged area was photobleached and fluorescence recovery was measured by time-lapse imaging for up to 2min post-irradiation. No significant differences were observed between the kinetics of recruitment of wild-type XLF, 4A-XLF or 6A-XLF at these shorter times after damage (Fig. 7B), however, when the kinetics of protein release were measured up to 2h after damage, dissociation of the 6A-XLF mutant was slightly slower than either wild-type or 4A-XLF (Fig. 7C). Thus, although phosphorylation of XLF is not required for recruitment to DSBs, it may promote the release of XLF from sites of DNA damage *in vivo*.

3.5. Phosphorylation of XLF is not required for survival after IR or for repair of DSBs *in vivo*

To determine whether phosphorylation of XLF was required for DSB repair *in vivo*, we first measured the kinetics of histone H2AX phosphorylation in XLF-deficient 2BN cells that had been transiently transfected with either pEGFP-wt-XLF or pEGFP-6A-XLF. Twenty-four hours after transfection, cells were exposed to 2Gy IR and the number of foci was measured in GFP-expressing cells. Similar numbers of foci were observed at early times after induction of damage (30–60min), indicating that mutation of the *in vivo* phosphorylation sites has no effect on the number of DSBs incurred in these cells (Fig. 8A). As expected, non-complemented 2BN cells showed a high level of residual γ -H2AX foci that persisted for at least 24 h after damage consistent with the presence of unrepaired DSBs (Fig. 8A and data not shown). However, reintroduction of either wt-XLF or 6A-XLF resulted in a decrease in the number of phospho-H2AX foci 1–4 h post-IR (Fig. 8A). Strikingly, the kinetics of H2AX phosphorylation in cells transfected with phosphorylation-defective XLF was indistinguishable from that of cells transfected with wt-XLF (Fig. 8A), indicating that phosphorylation of XLF is not required for DSB repair *in vivo*.

As a further test to determine whether phosphorylation of the C-terminus of XLF was required for function, 2BN cells were transiently transfected with either wt-XLF or 6A-XLF and survival after IR was determined using a colony formation assay. In a separate experiment, XLF was shown to be expressed for at least 6 days post-transfection (Supplementary Fig. 5). As expected, 2BN cells were highly sensitive to irradiation, whereas reintroduction of either wt-XLF or 6A-XLF resulted in a significant increase in survival after 1 Gy (Fig. 8B). Similar results were obtained after 2Gy IR (data not shown). Therefore, we conclude that phosphorylation of XLF is not required for cellular survival after IR.

4. Discussion

XLF is a newly discovered member of the NHEJ pathway. Since the protein kinase activity of DNA-PKcs is required for NHEJ, here we asked whether XLF is a substrate for DNA-PK. We show that XLF is phosphorylated *in vitro* by DNA-PK at two major sites, serines 245 and 251. Serine 245 is followed by leucine and therefore conforms to the non-SQ/TQ motif found in several other DNA-PK substrates (reviewed in [3]). In contrast, serine 251 is followed by a glutamine and is therefore an SQ site (Table 1). DNA-PK also phosphorylates serine 263 and threonine 266 and possibly threonine 223 *in vitro* (data not shown), however, these represent minor sites. All of the major *in vitro* phosphorylation sites are located in the C-terminal 60 amino acids of XLF (amino acids 240–299), in a region that is outside of the characterized head and stalk domains of XLF and is predicted to be largely unstructured [8,32]. Interestingly, the *in vitro* DNA-PK phosphorylation sites in the related protein XRCC4, which like XLF also contains characteristic head and stalk domains, are also located in the extreme C-terminus [18,19], in a region that is also predicted to be unstructured [18]. Similarly, the *in vitro* DNA-PK phosphorylation sites in both Ku70 and Ku80 are also located in regions that are predicted to be unstructured and/or are highly flexible in solution [33–35]. It is likely that such flexible regions may be more accessible to the active site of the large DNA-PKcs polypeptide.

We also show that XLF is highly phosphorylated *in vivo*, that *in vivo* phosphorylation of XLF is largely PIKK-dependent and that the identified *in vitro* sites, serines 245 and 251, represent the major *in vivo* phosphorylation sites in XLF. Serine 203, which does not conform to a known protein kinase consensus sequence, was also phosphorylated *in vivo*. Some phosphorylation of S132 was detected by MS/MS, but this was not confirmed by *in vivo* labeling (Table 1). Other SQ sites, such as serine 55 and serine 132, which are highly conserved in vertebrate species (see Fig. 1A in [8]), were not identified as major sites of

phosphorylation either *in vitro* or *in vivo*, possibly because they are located in globular domains that are either not accessible or are involved in dimerization or other protein–protein interactions.

Using phosphospecific antibodies, we show that serine 245 is phosphorylated *in vivo* in both unirradiated and irradiated transfected cells. Moreover, phosphorylation at this site was abrogated by inhibition of DNA-PK, suggesting that serine 245 is a DNA-PK-dependent phosphorylation site *in vivo*. In untransfected cells, endogenous XLF was only phosphorylated at serine 245 in irradiated cells, suggesting that the observed DNA-PK-dependent phosphorylation of XLF at this site in unirradiated, transfected cells might be a result of transient transfection. Serine 251 was also phosphorylated in irradiated, non-transfected human cells and was phosphorylated in response to IR in XLF-transfected human cells. Interestingly, phosphorylation at this site was ATM-dependent, indicating that both DNA-PKcs and ATM can phosphorylate XLF *in vivo*.

The C-terminal ~70 amino acids of human XLF have been predicted to contain DNA-binding activity [8]. Because the major *in vitro* phosphorylation sites in XLF were located in the C-terminal portion of XLF, we tested whether this region of XLF did indeed bind directly to DNA *in vitro* and whether phosphorylation by DNA-PK affected this interaction. Our experiments confirm that the C-terminus of human XLF does indeed possess the ability to bind to DNA, and as shown previously for full-length XLF, DNA binding of the C-terminal fragment was highly dependent on both the length of the DNA and protein concentration [12]. Interestingly, the C-terminal ~70 amino acids of the *Saccharomyces cerevisiae* homologue of XLF, Nej1p, also contains DNA binding activity and is phosphorylated in response to DNA damage *in vivo* [36]. However, phosphorylation of the C-terminal fragment of human XLF, at least at the sites identified in this study, did not have a significant effect on its ability to interact with DNA *in vitro* or *in vivo*.

We have also used three separate approaches to determine whether phosphorylation of XLF is required for DSB repair *in vivo*. Using a UV laser to introduce DNA damage in living cells, we show that phosphorylation of the C-terminus of XLF is not required for the initial recruitment of XLF to DSBs *in vivo*. These results are consistent with recent findings from Chen and colleagues that show that the protein kinase activity of DNA-PK is not required for recruitment of XLF to DNA after damage [21]. However, a modest decrease in the retention of the phosphorylation mutant at sites of laser-induced DNA damage was observed, suggesting that phosphorylation of XLF might contribute to the release of XLF from DSBs *in vivo*.

The kinetics of phosphorylation of histone H2AX is widely regarded as a surrogate for the kinetics of DSB repair *in vivo* [37]. Our results show that both wt-XLF and XLF in which up to six of the identified *in vivo* phosphorylation sites are mutated to alanine (6A-XLF) are able to complement the repair defect of XLF-deficient 2BN cells, and that the kinetics of γ -H2AX formation and dissipation are virtually identical in cells expressing either wt- or phosphorylation-defective XLF. Likewise, in colony formation assays, 6A-XLF was shown to complement survival after exposure to IR in 2BN cells. Together, these studies suggest that phosphorylation of XLF at the identified *in vivo* sites is unlikely to be required for DSB repair.

We also conclude that, like the related protein, XRCC4, which is highly phosphorylated by DNA-PK *in vitro*, XLF is not likely a physiologically relevant target of DNA-PK. However, we caution that these studies were carried out on transfected cells in which the expression of both wt- and phosphorylation-defective XLF protein was increased approximately eight-fold over the level of XLF in normal human fibroblasts (Supplementary Fig. 6). It is therefore

possible that over-expression could have masked subtle effects of phosphorylation on XLF function. It is also possible that by mutating multiple phosphorylation sites in XLF, differences in the effects of ATM-dependent versus DNA-PK-dependent phosphorylation events may have been missed. It is also possible that IR-induced phosphorylation of XLF affects other functions of the protein that were not examined here. However, we cannot exclude the possibility that, as has been suggested previously [38], not all IR-induced phosphorylation events have functional significance, and that some may simply be a consequence of exposing cells to large amounts of DNA damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Purified recombinant XLF is phosphorylated by DNA-PK *in vitro*. Full-length His-XLF was purified over NTA-agarose and incubated with purified DNA-PKcs and Ku as described in Section 2. (A) Coomassie stained SDS PAGE gel showing purified His-tagged XLF. Positions of molecular weight markers (in kDa) are shown on the left. (B) His-XLF was incubated with ^{32}P - γ -ATP without (–) and/or with (+) purified DNA-PK as indicated. Reactions were stopped by the addition of SDS and samples were analyzed by SDS PAGE followed by autoradiography. GST-XRCC4 was included as a positive control. The positions on the autoradiogram of autophosphorylated DNA-PKcs and phosphorylated Ku70 (indicated by the *), XRCC4 and XLF are shown. (C) His-XLF was incubated with DNA-PK, non-radioactive ATP and wortmannin (5 μM) as indicated, then analyzed by SDS PAGE followed by western blot using an antibody to phosphorylated SQ/TQ sites (Cell Signaling Inc). The position of phosphorylated DNA-PKcs and His-XLF are shown. (D) Serine/threonine to alanine mutations were generated in full-length GST-tagged XLF at serines 245 and 251 as indicated. The GST-tagged protein was cleaved using PreScission Protease (GE Healthcare), and the purified proteins were incubated without or with DNA-PK as indicated under standard assay conditions as described in Section 2 and analyzed by SDS PAGE and autoradiography. Upper panel, region of the Coomassie stained SDS PAGE gel corresponding to XLF; lower panel, region of the autoradiogram corresponding to XLF. Phosphorylation of the mutant proteins as a percentage of phosphorylation of wt-XLF is shown in the box. (E) Truncation mutations were generated in GST-XLF and the purified proteins, consisting of amino acids 1–239 and 240–299 as indicated, were phosphorylated by DNA-PK as described above. Upper panel, Coomassie stained SDS PAGE gel, lower panel, autoradiogram.

Fig. 2.

XLF-FLAG is phosphorylated in a DNA-PK and ATM-dependent manner *in vivo*. (A) HeLa cells were transfected with vector alone (lane 1) or XLF-FLAG as indicated (lanes 2–6). Samples in lanes 3–6 were irradiated (10 Gy) after addition of ³²P-inorganic phosphate. Where indicated, cells were incubated for 30 min with wortmannin (5 μM), the DNA-PK inhibitor NU7441 (10 μM), or the ATM inhibitor KU55933 (10 μM) in phosphate-free media as described in Section 2. Cells were then incubated with ³²P-inorganic phosphate and proteins were immunoprecipitated as described in Section 2. The upper panel shows the resulting autoradiogram and the lower panel the western blot for FLAG. Phosphorylation in each sample as a percentage of the phosphorylation in lane 2 is shown in the boxed region. (B) Cells were transfected with vector alone (lane 1), wt-XLF-FLAG (lane 2) or XLF-FLAG containing serine to alanine mutations at amino acids 132, 203, 245 and/or 251 (XLF-4A in figure) as indicated (lanes 3–7). Cells were labeled with ³²P-inorganic phosphate and immunoprecipitated as described in panel (A). The upper panel shows the resulting autoradiogram and the lower panel the western blot for FLAG. Quantitation was as described in panel (A).

Fig. 3.

DNA-PK and ATM are required for *in vivo* phosphorylation of XLF-FLAG on serine 245 and serine 251, respectively. (A) Cells were transfected with wt-XLF-FLAG (lanes 1–4) or XLF-FLAG with either serine 245 or 251 mutated to alanine [XLF(S245A) or XLF(S251A), respectively], (lanes 5 and 6). Cells were unirradiated (lane 1), or irradiated as indicated, then, after 2 h, cell extracts were immunoprecipitated with anti-FLAG agarose beads and probed with either phosphospecific antibodies to serine 245 or 251 or an antibody to FLAG as indicated. (B) Cells were transfected with either wt-XLF-FLAG (lanes 1–4) or phosphorylation mutant XLF-FLAG as indicated (lanes 5 and 6), irradiated at 5Gy, then analyzed at the times indicated by immunoprecipitation and western blot using antibodies to

phosphoserine 251 or FLAG as indicated. (C) Cells were transfected with wt-XLF-FLAG (lanes 1–7) and incubated with either the DNA-PK inhibitor NU7441 (lanes 3, 4 and 7) or the ATM inhibitor KU55933 (lanes 5–7) as indicated, or an equivalent volume of DMSO. The sample in lane 1 was not irradiated. Samples in lanes 2–7 were irradiated with 5Gy and two hours after irradiation, XLF was immunoprecipitated using anti-FLAG agarose beads and analyzed using phosphospecific antibodies to phosphoserine 245, phosphoserine 251 or FLAG as indicated.

Fig. 4.

Endogenous XLF is phosphorylated on serine 245 and 251 in irradiated human cells. XLF was partially purified from either unirradiated or irradiated human cells in the presence of protein phosphatase inhibitors as described in Supplementary materials and methods. XLF-containing fractions from either unirradiated cells (lanes 1 and 2) or irradiated cells (lanes 3 and 4) was analyzed by SDS PAGE and probed with phosphospecific antibodies to either serine 245 or serine 251 or total XLF as indicated. Where indicated (lanes 2 and 4, indicated by +), samples were incubated with λ phosphatase prior to SDS PAGE.

Fig. 5. The DNA-binding domain of XLF is located in the C-terminal 60 amino acids. Full-length GST-XLF was incubated with (A) 100, (B) 200 or (C) 300bp dsDNA and protein-DNA complexes were analyzed by EMSA as described in Section 2. The left hand figure in each panel shows lanes containing probe alone (lane 1), 0.012 (lane 2), 0.06 (lane 3), or 0.3 pmoles (lane 4) of purified Ku heterodimer. Lanes 6 and 8 contained 48 pmoles of either BSA or purified GST protein as indicated. The right hand panels contained 0 (lane 1), 6 (lanes 2, 6 and 10), 12 (lanes 3, 7 and 11), 24 (lanes 4, 8 and 12) or 48 (lanes 5, 9 and 13) pmoles of either full-length GST- XLF, GST-XLF₁₋₂₃₉ or GST-XLF₂₄₀₋₂₉₉, as indicated.

Fig. 6.

Phosphorylated C-terminal XLF retains its ability to interact with DNA. (A) Coomassie blue stained SDS gel showing purified GST-XLF₂₄₀₋₂₉₉ alone (control, c), after incubation with DNA-PKcs, Ku and DNA in the absence of ATP (mock, m) or the presence of ATP (phosphorylated, p). Phosphorylated protein was indicated by the supershifted bands (lane 3). (B) Non-phosphorylated, mock-phosphorylated or phosphorylated GST-XLF₂₄₀₋₂₉₉ was analyzed in EMSA assays for interaction with 300 bp dsDNA as described in Fig. 5. Lane 1 contained probe alone, lanes 2, 6 and 10 contained 6 pmoles of protein, lanes 3, 7 and 11 contained 12 pmoles of protein, lanes 4, 8 and 12 contained 24 pmoles and lanes 5, 9 and 13, contained 48 pmoles of protein. (C) Samples containing control (c), mock phosphorylated (m) and phosphorylated (p) GST-XLF₂₄₀₋₂₉₉ were prepared exactly as described above but 0.7 μ g phosphospecific antibody to serine 251 (lanes 2-4) or a non-specific (ns) IgG (lane 5) was added after addition of the DNA probe. Samples were incubated and analyzed by EMSA as in panel B.

Fig. 7.

Live cell imaging of wild-type and phospho-mutant XLF. (A) Accumulation of EGFP-tagged wild-type and phosphomutant XLF at laser-induced DSBs. EGFP-XLF and EGFP-XLF containing mutations at either four or six of the phosphorylation sites (4A mutant and 6A mutant, respectively) were expressed in U2OS cells. DSBs were induced in the defined area of the nucleus by a pulse of laser microbeam (shown by arrows), and accumulation of EGFP-XLF was monitored by time-lapse imaging. (B) FRAP analysis of EGFP-XLF at DSBs. Following induction of DSBs by laser micro-irradiation, cells were incubated to allow maximum accumulation of EGFP-XLF at DSBs. The damaged area in the nucleus was photobleached, and the recovery of the fluorescence signal was then monitored by time-lapse imaging. Mean values of relative fluorescence intensities at each time point were calculated from 10 independent measurements. (C) Two hour time-course of EGFP-XLF accumulation at DSBs. Mean values of the relative fluorescence at each time point were calculated from 10 independent measurements.

Fig. 8.

Phosphorylation mutant 6A-XLF complements DSB repair defects in XLF-deficient 2BN cells. (A) 2BN cells were transiently transfected with pEGFP-wt-XLF (solid line, closed squares) or pEGFP-6A-XLF (dashed lines, open circles) and cells were probed for γ -H2AX as described in Section 2. γ -H2AX foci were counted in GFP-labeled cells and compared to those in non-GFP labeled cells (solid line, open squares). The results are plotted as average number of foci per cell. (B) 2BN cells were transiently transfected with vector alone, pEGFP-wt-XLF, or pEGFP-6A-XLF. Cells were irradiated (1 Gy) 24 h after transfection, then seeded in triplicate and colonies were counted after crystal violet staining 14 days later. XLF protein levels were constant for at least 6 days post-transfection (Supplementary Fig. 5). Surviving fraction was calculated from number of colonies counted divided by numbers of cells seeded $\times$ plating efficiency. Results are representative of three separate experiments.

Table 1
Summary of identified *in vitro* and *in vivo* phosphorylation sites in XLF

Amino acid	Amino acid sequence	<i>In vitro</i> phosphorylation sites in XLF	<i>In vivo</i> phosphorylation sites in XLF
Serine 55	TSVV <u>S</u> QRAK	DNA-PK (minor, <10%)	Not detected
Serine 132	PSLV <u>S</u> QHLL	DNA-PK (minor, <10%)	<i>In vivo</i> (MS); minor by <i>in vivo</i> labeling
Serine 203	PEAC <u>S</u> IGDG	Not detected	<i>In vivo</i> (MS; <i>in vivo</i> labeling)
Threonine 223	AVT <u>T</u> QEVQ	DNA-PK (minor)	Not detected
Serine 245	SNSA <u>S</u> LQGI	DNA-PK (major, ~60%)	<i>In vivo</i> (MS; <i>in vivo</i> labeling), DNA-PK-dependent (ps-Ab)
Serine 251	QGID <u>S</u> QCVN	DNA-PK (major, ~75%)	<i>In vivo</i> (MS; <i>in vivo</i> labeling;), ATM-dependent (ps-Ab)
Serine 263	QLV <u>S</u> SAPTL	DNA-PK (minor)	<i>In vivo</i> (MS)
Threonine 266	SSAP <u>T</u> LSAP	DNA-PK (minor)	Indicated <i>in vivo</i> (MS)

MS: identified by mass spectrometry; ps-Ab: confirmed by phosphospecific antibodies. The phosphorylated amino acid in each sequence (column two) is indicated in bold and underlined.