

XLF-Cernunnos promotes DNA ligase IV–XRCC4 re-adenylation following ligation

Enriqueta Riballo, Lisa Woodbine, Thomas Stiff, Sarah A. Walker,
Aaron A. Goodarzi and Penny A. Jeggo*

Genome Damage and Stability Centre, University of Sussex, East Sussex, BN1 9RQ, UK

Received October 14, 2008; Revised November 7, 2008; Accepted November 11, 2008

ABSTRACT

XLF-Cernunnos (XLF) is a component of the DNA ligase IV–XRCC4 (LX) complex, which functions during DNA non-homologous end joining (NHEJ). Here, we use biochemical and cellular approaches to probe the impact of XLF on LX activities. We show that XLF stimulates adenylation of LX complexes de-adenylated by pyrophosphate or following LX decharging during ligation. XLF enhances LX ligation activity in an ATP-independent and dependent manner. ATP-independent stimulation can be attributed to enhanced end-bridging. Whilst ATP alone fails to stimulate LX ligation activity, addition of XLF and ATP promotes ligation in a manner consistent with XLF-stimulated readenylation linked to ligation. We show that XLF is a weakly bound partner of the tightly associated LX complex and, unlike XRCC4, is dispensable for LX stability. 2BN cells, which have little, if any, residual XLF activity, show a 3-fold decreased ability to repair DNA double strand breaks covering a range of complexity. These findings strongly suggest that XLF is not essential for NHEJ but promotes LX adenylation and hence ligation. We propose a model in which XLF, by *in situ* recharging DNA ligase IV after the first ligation event, promotes double stranded ligation by a single LX complex.

INTRODUCTION

DNA non-homologous end-joining (NHEJ) is the major mechanism for the repair of radiation induced DNA double strand breaks (DSBs) in mammalian cells. Cell lines lacking NHEJ components are exquisitely radiosensitive and DSB repair defective (1,2). NHEJ also functions to effect rearrangements at site-specific DSBs introduced during V(D)J recombination (3). Consequently, viable

mice lacking NHEJ proteins show severe combined immunodeficiency (SCID). Patients deficient in NHEJ components have also been described. Most patients show varying degrees of combined immunodeficiency, microcephaly and developmental delay and cell lines derived from them display radiosensitivity, which leads to the classification of radiosensitive-(RS)-SCID (4–7). One patient received radiotherapy and dramatically over-responded to treatment demonstrating clinical radiosensitivity (8,9).

The first step of NHEJ is binding of the heterodimeric Ku protein to double stranded (ds) DNA ends. Crystallography studies on Ku have shown that it can encircle DNA with a central core of sufficient diameter to allow the threading and translocation of Ku onto dsDNA (10). Once bound to DNA, Ku recruits the DNA–PK catalytic subunit (DNA–PKcs), creating the active DNA–PK holoenzyme (1,2). Increasing evidence suggests that DNA–PK undergoes autophosphorylation, which regulates the process and/or facilitates the recruitment of additional proteins required for end processing, such as Artemis (11,12). The assembled DNA–PK complex facilitates the recruitment of a ligation complex encompassing XRCC4 and DNA ligase IV (13,14).

XLF-Cernunnos, hereafter called XLF, was identified via the analysis of a class of RS-SCID patients with features closely resembling those of LIG4 syndrome patients, a disorder caused by mutations in DNA ligase IV (6,7,15). This strongly suggested that XLF is a component of the NHEJ machinery, which is substantiated by the finding that XLF interacts with XRCC4 and is co-recruited with NHEJ components to DSBs (15,16). Furthermore, it has recently been reported that Ku plays a role in recruiting XLF to DSBs (17). Interestingly, XLF was predicted, and has now been shown, to have a structure similar to, but distinct from, that of XRCC4 with an N-terminal globular head domain and C-terminal coiled coil structure, which is shorter than that in XRCC4 and directed towards the N-terminal region (15,18,19). Furthermore, XLF represents the mammalian homologue of the yeast Nej1p protein, a factor regulating NHEJ in yeast (20). Since both

*To whom correspondence should be addressed. Tel: +44 1273 678 482; Fax: +44 1273 678 121; Email: p.a.jeggo@sussex.ac.uk

LIG4 and *XLF*-deficient cell lines are proficient in Ku end-binding activity and display normal DNA-PK activity, the available data provides strong evidence that XLF is a further factor required for ligation during NHEJ (6,15). The co-immunoprecipitation of XLF with LX has led to the suggestion of a tripartite XLF–XRCC4–ligase IV complex, although a more recent study has suggested that XLF does not impact on LX association or DSB recruitment (16). XLF has been reported to promote LX ligation potentially by enhancing end-bridging because of its ability to bind DNA (15,21,22). However, multiple proteins are able to promote end-bridging *in vitro* including DNA-PKcs and Ku, and DNA ends might also be partially tethered by higher order DNA structure (23–25). XLF has also been reported to be involved specifically in the ligation of mismatched and noncohesive DNA ends (21,26). Here, we examine in further detail the impact of XLF on LX activities.

The ligation reaction involves an initial charging step generating a DNA ligase IV–adenylate complex followed by transfer of the AMP moiety to the 5′ phosphate on DNA creating a DNA–adenylate complex and releasing uncharged DNA ligase IV, which needs to be re-adenylated for subsequent ligation. A recent study characterizing LX based on its nick ligation activity highlighted that, in contrast to other DNA ligases, LX re-adenylation following ligation proceeds very slowly and is rate limiting *in vitro* (27). In support of this, we have never observed any stimulation of LX-mediated ds ligation by ATP (28).

Here, we have used biochemical and cellular approaches to probe the role of XLF in NHEJ. We provide the first demonstration that XLF stimulates LX adenylation following ligation, which enhances the ability of LX complexes to stimulate ds ligation in the presence of ATP. To gain insight into the *in vivo* impact of XLF function, we examined two lines harbouring mutations in XLF. We demonstrate that 2BN cells have little, if any, residual XLF function but manifest only a 3-fold decrease in rejoining DSBs covering a range of complexities. Our findings provide evidence that XLF stimulates but is non-essential for NHEJ.

MATERIALS AND METHODS

Cell culture

Primary fibroblasts, 1BR3 (control), 2BN and F07/402 (*XLF*-deficient), 180BR and 495GOS (*LIG4*-deficient), hTERT immortalized fibroblasts, 48BRhT (control), 2BNhT, 180BRhT, 495GOSHt, and SV-40 transformed human fibroblasts, MRC5BIVA, were grown in minimal essential medium (MEM) as described previously (6,29). Human PreB Nalm6 (wild type) and N114P2 (*Lig4* null) were grown in RPMI medium supplemented with 10% foetal calf serum (FCS), penicillin and streptomycin. Chinese hamster ovary (CHO) cells, AA8 (wild type) and XR-1 (*XRCC4* null) and mouse embryonic fibroblasts (MEFs) MEF22 and *Lig4*^{-/-}*p53*^{-/-} were grown in MEM, supplemented with 10% FCS, penicillin and streptomycin.

XLF was cloned into pCI neo-n-FLAG and c-HA, originally from Promega (Madison, USA) and modified by Dr E. Taylor. To introduce the I11nsT mutation into *XLF* cDNA, QuickChange^R XL Site-Directed Mutagenesis kit from Stratagene was used (Cambridge, UK). MRC5BIVA were transfected using Fugene (Roche, Burgess Hill, UK).

Neocarzinostatin and Etoposide (Sigma-Aldrich, (Dorset, UK) were added at the indicated concentrations and Etoposide was removed after 1 h. AMPCPP was from Jena Bioscience GmbH (Jena, Germany).

Immunofluorescence, immunoblotting and antibodies

Immunofluorescence analysis was carried out as previously described (30). Whole cell extracts for immunoblotting and immunoprecipitations were prepared as described previously (15). α -DNA ligase IV and α -XRCC4 rabbit antibodies were from Serotec (Oxford, UK). α -XLF rabbit antibody was generated against bacterially expressed XLF by Eurogentec (Seraing, Belgium). α -Ku70 goat antibody and α -cMYC (9E10) were from Santa Cruz Biotechnology (Santa Cruz, CA). ANTI-FLAG M2 monoclonal antibody was purchased from Sigma-Aldrich (Poole, UK). 6xHIS monoclonal antibody came from Clontech (Palo Alto, CA). Anti-HA antibody (12CA5) was from Cancer Research UK. α - γ -H2AX mouse antibody was from Upstate Technology (Buckingham, UK). Anti-rabbit, anti-mouse and anti-goat secondary antibodies were purchased from Dako (Glostrup, Denmark).

For *in vitro* transcription–translation system (TNT), pcDNA3-n-His-*LIG4* (9), pCI neo-n-Myc-*XRCC4* and pCI neo-n-FLAG-*XLF* (this work) were expressed using Promega TNT T7 Quick couple transcription/translation system (Madison, USA).

Purification of DNA ligase IV–XRCC4 complexes, DNA-PK and XLF

The expression and purification of DNA ligase IV–XRCC4 complexes and XLF was performed as described previously (22,31). For XLF purification, the bacterial pellet was lysed in 50 mM Tris pH 8.0, 5 mM EDTA, 400 mM NaCl, 1 mM DTT, 1 mM PMSF and 1% Triton X-100, followed by sonication and clarification by centrifugation. GST-XLF was bound to GST beads and washed with lysis buffer. When required, the GST tag was removed by thrombin treatment. The protein was concentrated using Vivascience 0.5 ml column (Sartorius). DNA-PK was purified as described previously (32).

Adenylation and ligation

Adenylation and ligation were performed as described previously (28,31). Briefly, for adenylation reactions, LX complexes were incubated in the presence of 5 mM disodium pyrophosphate for 15 min at room temperature in 20 mM Tris–HCl, pH 8.0, 50 mM NaCl. Pyrophosphate was removed using a Vivascience 0.5 ml column (Sartorius). The complexes were incubated in the presence of purified XLF or 0.1 μ g/ml of BSA and α -³²P-ATP (2 μ Ci, 66 nM final concentration) (GE Healthcare, Buckinghamshire, UK). For ligations, a 445-bp DNA

fragment was produced from Bluescript plasmid (Stratagene) by digestion with PstI and AflIII (New England Biolabs). For the reactions, the indicated amount of protein complexes were incubated for the indicated times in 20 μ l of ligation reaction (50 mM triethanolamine, pH 7.5, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 9% polyethylene glycol) with the labelled substrate and in the presence or absence of 2 mM ATP or AMPCPP.

Bridging assay for the association of two DNA molecules

This assay was performed as described previously (24,25). The DNA fragment was the 445-bp pBluescript cohesive fragment used for ligation analysis. This substrate was labelled with a biotin group using the BrightStar Psoralen-Biotin Nonisotonic labelling Kit (Ambion, Warrington, UK). The binding buffer was the one used for ligation (50 mM triethanolamine, pH 7.5, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin) without polyethylene glycol and supplemented with 40 mM KCl and 1 mM ATP. The reaction was carried out at room temperature for 15 min.

siRNA transfection

Cells were untreated or transfected twice with scramble or XLF siRNA (0.2 μ M) from Dharmacon SMART pool (Dharmacon, USA) using HiPerFect transfection reagent (QIAGEN, Hilden, Germany).

RESULTS

XLF stimulates ligase IV re-adenylation following ligation

The first step of the ligation reaction is binding of AMP to the active site lysine of DNA ligase IV generating an adenylated ligase with the release of pyrophosphate. Firstly, we examined whether XLF impacts upon LX adenylation activity. Since we were unable to purify a co-expressed trimeric complex because of the weak association of XLF with LX at greater than physiological salt concentrations (see below), we used separately purified, bacterially expressed XLF and insect cell-expressed LX. LX is expressed in insect cells as a pre-adenylate complex and assessment of adenylation activity is normally carried out following de-adenylation by inorganic pyrophosphate (PPi), which causes AMP release. Incubation of the de-adenylated complex with radioactively labelled α -ATP generates a labelled AMP-LX (adenylate) complex that can be detected on an SDS-PAGE gel. Using 5 pmol of XLF and 1 pmol of LX, we observed 2–3-fold stimulation in the rate of adenylation over 20 min (Figure 1A; gels shown in Supplementary Figure S1A). A similar degree of stimulation was observed using between 5 and 30 pmol XLF (data not shown).

We and others have previously observed that re-adenylation of LX following ligation is very inefficient (27,28). Therefore, we considered the possibility that XLF might play a role in recharging LX following ligation. We first examined the impact of ligatable DNA on adenylation using LX complexes previously de-adenylated by PPi

treatment. We utilized a 445-bp pBluescript fragment, which we have previously used in ds ligations (28), and observed that the presence of DNA decreases adenylate complex formation regardless of the presence of XLF (Figure 1B and Supplementary Figure S1B). This suppressive impact was more marked at later times consistent with ligation causing LX de-adenylation thereby reducing the overall amount of adenylate complex observed.

To determine whether XLF stimulates re-adenylation following ligation, we adapted our assay to examine adenylate complex formation without prior de-charging of LX complexes by PPi treatment. Instead, we examined re-adenylation of LX following de-charging by ligation. To achieve this, we allowed ligation to take place in the absence of ATP, with or without XLF, during a 15 min pre-incubation step prior to addition of radiolabelled α -ATP to monitor adenylate complex formation (Figure 1C and Supplementary Figure S1C). XLF promoted LX adenylation following incubation in the presence of DNA, while such activity occurred slowly in the absence of XLF, providing the first demonstration that significant re-adenylation of LX can occur following ligation. To verify that ligation had taken place, we monitored ligation during the pre-incubation phase in the absence of ATP and subsequently when ATP (with or without XLF) was added (Figure 1D). We observed ligation products during the pre-incubation reaction consistent with de-adenylation occurring as a consequence of ligation. However, ligation peaked at 15 min and further stimulation following addition of ATP was not observed either in the presence or absence of XLF. This is likely due to the fact that only a small fraction of LX complexes are recharged during the reaction since optimization of our ability to detect LX-³²P-adenylate complex formation required using radiolabelled ATP without added cold ATP. Thus, the ATP concentrations (66 nM) are likely below the optimal K_m for ATP for DNA ligase IV. The presence of XLF during the 15 min pre-incubation step did not increase either the rate of LX adenylation (Figure 1C) or the level of ligation (data not shown), suggesting that XLF may not enhance ligation without prior de-adenylation of LX. However, in this experiment an excess of LX to substrate (5:1) was utilized, potentially limiting any ability to detect XLF stimulatory activity. We, therefore, modified the assay to examine the impact of XLF on ligation under LX rate-limiting conditions (1:4 ratio of LX to DNA ends) but still failed to observe any marked stimulation (Figure 1E). These findings show that while XLF does not markedly stimulate ligation during the pre-incubation phase, it markedly enhances the rate of adenylation. Thus, we demonstrate that there is an impact on LX re-charging that cannot be attributed to any marked impact of XLF on ligation. We also examined the stimulation of adenylation in the presence of non-ligatable DNA. We used a blunt ended DNA substrate since blunt ended DNA is a poor substrate in our *in vitro* ligation reaction. Blunt-ended DNA failed to stimulate either adenylation or ligation activity (Supplementary Figure S2). Thus, we conclude that the stimulation of adenylation by XLF in this assay follows de-adenylation by ligation.

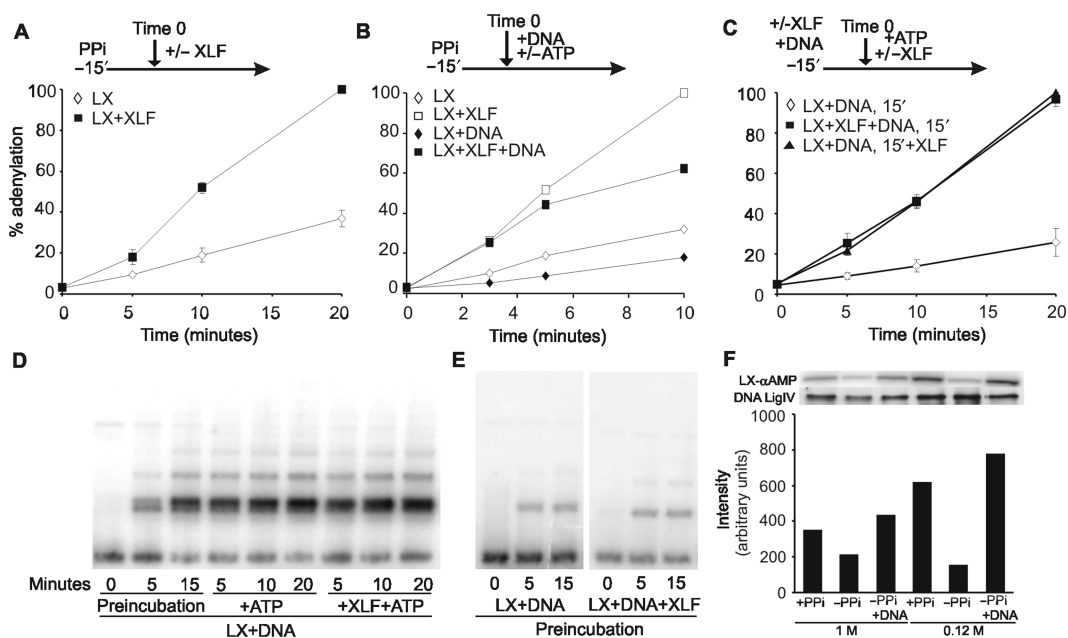


Figure 1. XLF stimulates ligase IV re-adenylation following ligation. (A) LX was treated with pyrophosphate (PPi) for 15 min at room temperature. Following dialysis, 1 pmol LX was incubated with α - 32 P-ATP with or without 5 pmol XLF. XLF stimulated LX adenylation activity 2.5-fold. Results and error bars are the mean and SD of two experiments. (B) Following PPi treatment and dialysis, 1 pmol LX was incubated with α - 32 P-ATP, with or without 30 pmol XLF and 0.2 pmol DNA ends. (C) One picomole LX was incubated with 0.2 pmol DNA ends for 15 min to allow ligation and hence de-adenylation. α - 32 P-ATP was then added with or without 5 pmol XLF. XLF enhanced adenylation 3-fold. Whether XLF was present or absent during the pre-incubation step did not change the level of adenylation. Results are the mean and SD of two experiments. (D) Samples treated as in (C) were examined for ligation by gel electrophoresis. Ligation occurred during the preincubation step and peaked by 15 min. (E) Total 0.2 pmol LX were incubated with 0.8 pmol DNA ends with or without 1 pmol XLF without ATP for 15 min. Samples were examined for ligation. XLF did not impact upon the level of ligation. (F) Two milligrams WCE from human pre-B control cells (Nalm6) were immunoprecipitated with α -XRCC4 antibodies, split in half and washed either in buffer containing 1 or 0.12 M NaCl. Samples were treated with or without PPi in the presence or absence of \sim 35 pmol DNA ends and were examined for adenylation activity. LX- α AMP is the adenylated LX complex. Immunoprecipitated samples were subjected to immunoblot analysis using α -DNA ligase IV antibodies. The graph shows arbitrary units of intensity of adenylation normalized to protein levels.

Finally, to examine whether XLF stimulates LX adenylation using physiologically relevant ratios of XLF and LX, we used α -XRCC4 antibodies to immunoprecipitate LX-XLF complexes from extracts from a human pre-B cell line using 0.12 M NaCl and employed a high salt (1 M NaCl) wash to discharge XLF (see below). This allows a comparison of the impact of XLF in the same cellular background. De-adenylation of LX was carried out by PPi treatment or by incubation with DNA (445-bp pBluescript fragment). XLF stimulated LX adenylation activity following PPi treatment (+PPi, 1 M NaCl versus +PPi, 0.12 M NaCl) but strikingly, stimulation was even greater when LX was de-charged by DNA (+DNA, 1 M NaCl versus +DNA, 0.12 M NaCl) (Figure 1F). Thus, even though our biochemical studies require a ratio of XLF to LX greater than one, potentially due to a percentage of inactive XLF in the XLF preparations, the ratio of XLF to LX obtained by co-immunoprecipitation was also able to stimulate LX re-adenylation linked to ligation, confirming our *in vitro* findings.

ATP-dependent and independent stimulation of LX double-stranded ligation by XLF

Previous studies have shown that XLF stimulates LX ds ligation activity (21,22). Our finding that XLF stimulates

LX recharging following ligation predicted that XLF stimulation of ligation should be ATP-dependent. Although we were unable to observe XLF stimulation of ligation in the experiments described above (Figure 1D), this was potentially attributable to the low ATP concentration utilized in the assay. We, therefore, examined the impact of concentrations of ATP in the physiological range (2 mM) on the ability of XLF to stimulate LX ds ligation. First, we titrated the amount of XLF stimulating 125 fmol LX, which effects only a low level of ligation and observed a broad peak of stimulation from 2.5 to 30 pmol XLF, which varied slightly with individual LX preparations (Figure 2A). We next examined the ability of 17 pmol XLF to stimulate ligation effected by differing amounts of LX in the presence or absence of ATP (Figure 2B). In the absence of XLF, little ds ligation was observed. In agreement with previous findings and the discussion above, addition of ATP alone did not stimulate LX activity consistent with the dramatically reduced ability of LX to undergo re-adenylation post ligation (Figure 2B, compare left and right panels) (27,28). XLF provided some stimulation in the absence of ATP but further stimulation was observed in the presence of ATP (Figure 2B, right panel). Significantly, when high amounts of LX were employed (50 fmol) with or without ATP, XLF provided no further stimulation, consistent with the notion that LX

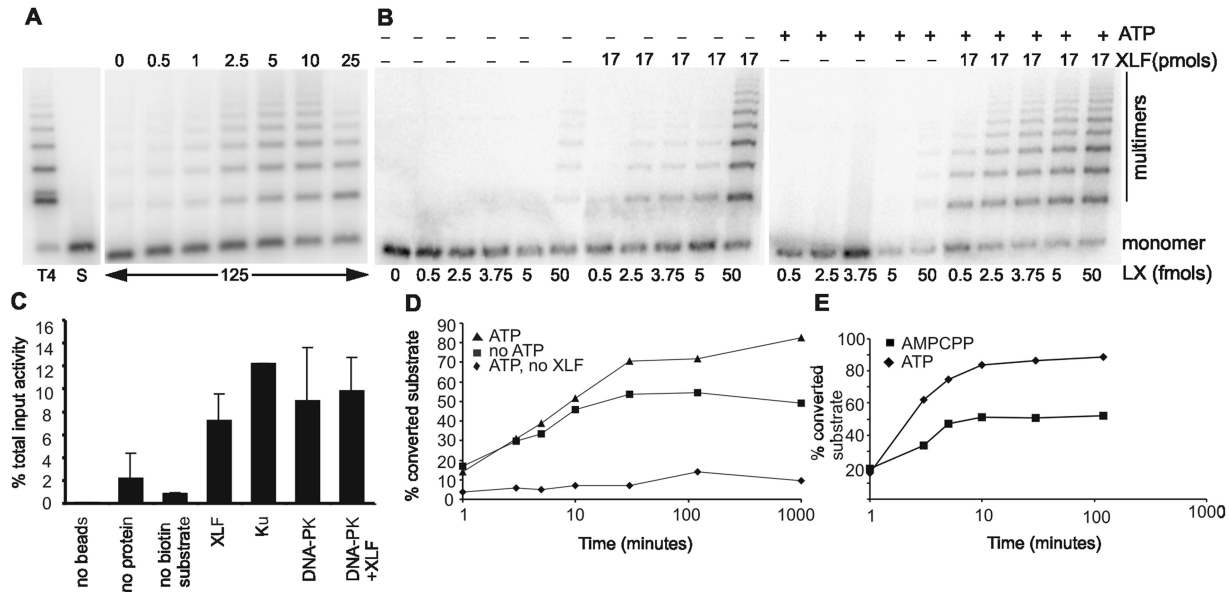


Figure 2. XLF stimulates LX double stranded ligation in an ATP-dependent and independent manner. (A) On the left panel, substrate ligated by T4 and unligated substrate were run as controls. On the right panel, 125 fmol LX was incubated with 5'-³²P-end-labelled DNA fragment (445 bp) and increasing amounts of XLF. Ligation products were analysed by gel electrophoresis. XLF stimulated ligation. (B) Increasing amounts of LX complexes were incubated with or without 17 pmol GST-XLF in the absence (left panel) or presence (right panel) of 2 mM ATP and 5'-³²P-end-labelled substrate. (M, monomer) No multimers formed in the absence of XLF. XLF stimulated LX ligation in the absence of ATP but further stimulation was observed in the presence of ATP. (C) Twenty-five femtomoles 5'-³²P-end-labelled DNA fragment were incubated with 100 fmol biotinylated substrate at RT for 15 min and the presence of co-associated radioactive substrate estimated. GST-XLF (17 pmol) shows the same level of bridging as 9.7 pmol of DNA-PK holoenzyme or 1.3 pmol of Ku. (D) Five femtomoles LX complexes were incubated with 15 pmol XLF and 5'-³²P-end-labelled DNA fragment. Ligation was estimated at time points indicated. No ligation occurred in the absence of XLF. XLF stimulated ligation in the absence of ATP but the maximum level of ligation was increased in the presence of XLF and ATP. (E) Five femtomoles LX complexes were incubated with 10 pmol XLF and 5'-³²P-end-labelled DNA fragment. XLF could not stimulate the ligation reaction in the presence of AMPCPP to the level reach in the presence of ATP.

is not rate limiting and that ds ligation can occur by two independent ligation events. These findings provide strong evidence that XLF can stimulate adenylation under physiological ATP concentrations and moreover, that such stimulation can enhance ds ligation.

It has been suggested that XLF might function as a bridging factor to facilitate alignment and rejoining of ds DNA ends (22,33). To examine this, we used a pull-down assay in which the 445-bp duplex substrate used for ligation was biotin labelled and bound to magnetic streptavidin beads and incubated together with XLF and ³²P-end-labelled substrate. Intermolecular substrate association was assessed by monitoring the ability of streptavidin beads to retain the ³²P-labelled duplex following multiple washes (Figure 2C). XLF enhanced the recovery of radiolabelled DNA associated with beads demonstrating that it facilitates DNA end-bridging. As a control, DNA-PK holoenzyme (DNA-PKcs-Ku80/70), which also promotes end-bridging, caused a similar increase in duplex-bridging activity, and XLF and DNA-PK holoenzyme together did not show any additive effect (32). These findings support the notion that XLF promotion of ligation in the absence of ATP might be attributable to its ability to promote end-bridging. The experiments carried out in Figure 1D used a low XLF to LX molar ratio (~5:1), optimized to observe the stimulatory effect of XLF on LX adenylation. In contrast, the ligation experiments in Figure 2B used a higher molar ratio of XLF to

LX (~75:1). Since the ratio of LX to DNA ends remains essentially the same (~5:1 versus ~2:1, respectively), it is possible that the higher level of XLF is required to promote DNA end bridging. Multiple proteins promote end-bridging and stimulate *in vitro* ligation reactions. Since XLF interacts with LX, we considered that this might provide an added impact on ligation *in vivo* but was unlikely to represent the unique function of XLF in NHEJ.

The ATP-dependent impact of XLF is unique, however, and consistent with the notion that XLF promotes LX recharging. To further examine this, we looked at the impact of XLF on the kinetics and maximal level of ligation with or without ATP (Figure 2D). In the absence of XLF (diamonds), there is hardly any ligation going on. In the absence of ATP, stimulation of ligation by XLF occurred within the first few minutes and little further stimulation was observed (squares). ATP together with XLF also enhanced the ligation within the first few minutes (triangles) but the maximal level obtained was higher than that observed in the absence of ATP. We conclude that XLF stimulates ds LX ligation activity in two ways, one being ATP-independent and the other ATP-dependent. In the presence of ATP, XLF enhances the maximal level of ligation consistent with our findings that it promotes recharging of LX following ligation. We also used an ATP analogue, AMPCPP, which cannot be hydrolysed to AMP and thus cannot generate an LX-adenylate complex. The level of stimulation of ligation observed in the

presence of AMPCPP was less than that observed in the presence of ATP (Figure 2E) and, interestingly, was similar to that observed in the absence of ATP (Figure 2D).

XLF is dispensable for DNA ligase IV stability and is a weaker binding partner of the stable LX complex

To examine XLF function *in vivo*, we used the 2BN cell line, which was derived from a patient with SCID with a single base pair (+T) insertion at position 11 (11insT) in the first coding exon of *XLF* (6,15). The insertion introduces a frameshift and creates a termination codon at position 139. Thus, the mutant protein only shares the first four amino acids with the wild-type protein. Using polyclonal antibodies raised to bacterially expressed, full-length, human XLF (α -XLF), we were unable to detect any residual XLF protein in 2BN cell extracts by immunofluorescence or immunoblotting (Figure 3A and B) despite a strong signal obtained with control cell extracts consolidating previous analysis of 2BN cells using a distinct α -XLF antibody (15). We were also unable to detect any smaller XLF fragments in 2BN cell extracts providing no evidence for translational re-initiation (data not shown). Given the lack of any significant XLF expression in 2BN cells, we examined the interdependence of XLF, XRCC4 and DNA ligase IV expression on protein stability. Immunoblotting and immunofluorescence showed that XRCC4 and DNA ligase IV are expressed normally and localize to the nucleus in 2BN cells (Figure 3A and B). In contrast, XR-1 cells, which lack XRCC4, express low levels of DNA ligase IV, as reported previously (Figure 3B) (34). Moreover, XRCC4 expression in a *LIG4* null cell line, N114P2, is approximately one third reduced compared to the parent line, Nalm6 (Figure 3B). XLF was expressed normally in DNA ligase IV-defective N114P2 cells. XLF expression in XR-1 cells could not be assessed since the α -XLF antibodies do not cross-react with hamster XLF. Collectively, these findings demonstrate that, whereas XRCC4 and DNA ligase IV are co-dependent for normal expression, XLF is dispensable for the stability of both XRCC4 and DNA ligase IV. Conversely, DNA ligase IV is dispensable for XLF stability and nuclear localization.

As the tightly associated LX complex is stable under high ionic strength, we examined the stability of the XLF-LX complex. We examined the impact of ionic strength on the interaction between DNA ligase IV, XRCC4 and XLF using cell extracts derived from MRC5BIVA cells expressing N-terminal FLAG-tagged XLF (F-XLF). α -FLAG antibodies efficiently co-immunoprecipitated DNA ligase IV under physiological salt conditions (0.12 M NaCl) but the interaction was substantially reduced at 0.5 M NaCl and essentially abolished at 1 M NaCl (Figure 3C). This contrasts with the strong interaction between XRCC4 and DNA ligase IV which is stable even at 1 M NaCl (35). We also examined interaction following expression of S^{35} -methionine radiolabelled, tagged proteins (HIS-LigIV, MYC-XRCC4 and FLAG-XLF) in a transcription-translation system (Figure 3D). α -MYC antibodies efficiently co-immunoprecipitated XLF at 0.12 mM NaCl but not at 1 M NaCl.

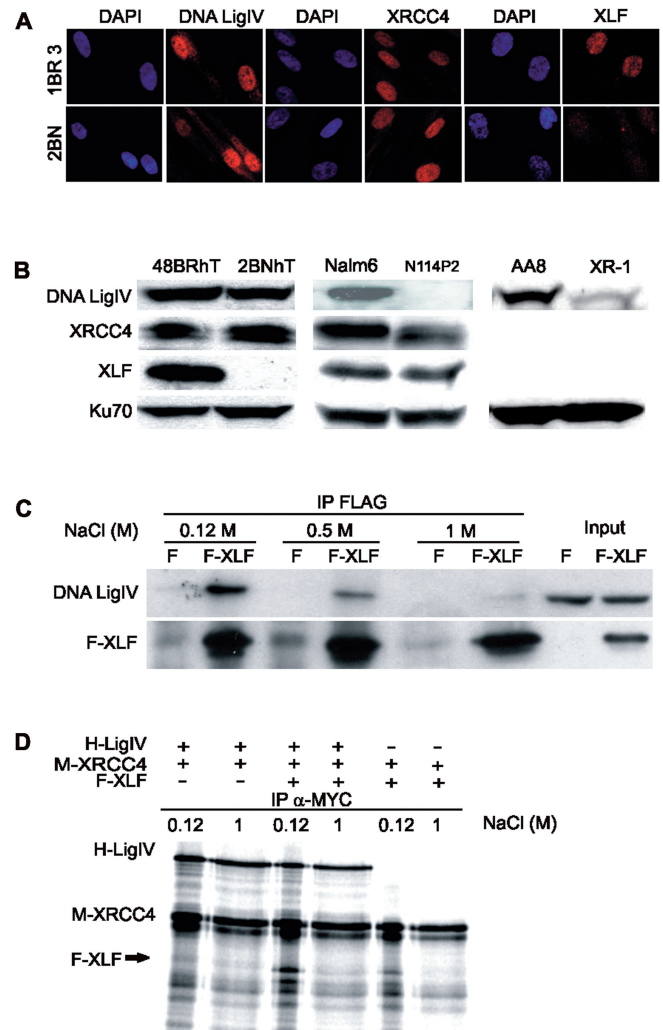


Figure 3. XLF is dispensable for DNA ligase IV stability and can be dissociated from the stable LX complex. (A) 1BR3 and 2BN cell lines were analysed by immunofluorescence using antibodies against DNA ligase IV, XRCC4 and XLF. 2BN cells lack XLF but express normal levels of ligase IV and XRCC4. (B) *Left panel:* 100 μ g of whole cell extract (WCE) from control (48BRhT) and XLF-deficient (2BNhT) were analysed by immunoblotting with α -DNA ligase IV, α -XRCC4 and α -XLF antibodies. Immunoblots were re-probed with α -Ku70 antibody as a loading control. (A) and (B) show that 2BN cells express normal levels of DNA ligase IV and XRCC4 but not XLF. *Middle panel:* 15 μ g of human control (Nalm6) and DNA ligase IV null pre-B cells (N114P2) were examined by immunoblotting. XRCC4 protein levels were reduced in the N114P2 cells compared to control cells but the levels of XLF were normal. *Right panel:* 60 μ g of WCE from CHO control (AA8) and XRCC4-deficient (XR1) cells were analysed by immunoblotting. (C) MRC5BIVA cells were transiently transfected with N-terminal FLAG-tagged XLF (F-XLF) or empty vector (F) and 500 μ g of WCE from both conditions were incubated with α -FLAG antibodies. The immunoprecipitates were analysed with α -DNA ligase IV antibodies after washing with buffer containing 0.12, 0.5 or 1 M NaCl. The blots were also analysed with α -FLAG antibodies to check for the presence of FLAG-tagged XLF (bottom panel). The interaction between XLF and LX is disrupted at 1 M NaCl. (D) HIS-LigIV, MYC-XRCC4 and FLAG-XLF were expressed individually in an *in vitro* transcription and translation (TNT) system in the presence of S^{35} -methionine, mixed and immunoprecipitated using α -MYC antibodies. Immunoprecipitates were washed in 0.12 or 1 M NaCl. The samples washed with 1 M NaCl have distorted (wider lanes). No FLAG-XLF was detected from immunoprecipitates washed at 1 M NaCl.

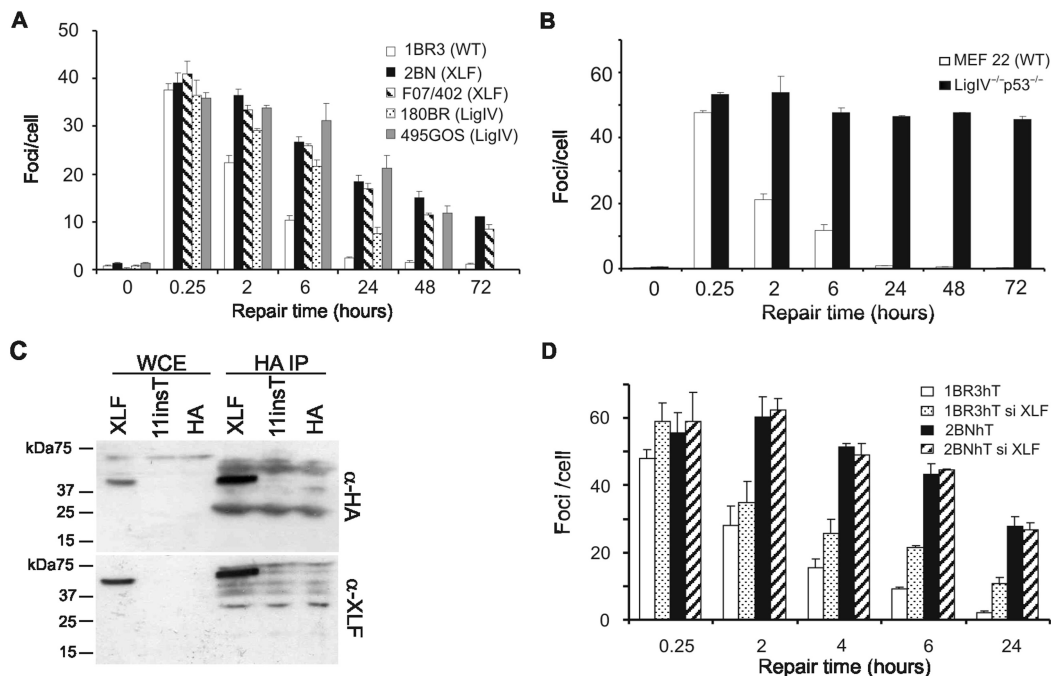


Figure 4. 2BN cells show reduced DSB rejoining. (A) Human primary fibroblasts from control (1BR3), XLF-deficient (2BN and F07/402) and LIG4 syndrome patients (180BR and 495GOS) were irradiated (3 Gy) and the rate of loss of γ -H2AX foci enumerated using α - γ -H2AX antibody. Results are the mean and SD of at least three experiments. The rate of DSB repair in 2BN and F07/402 cells is similar to that of LIG4 syndrome cell lines. (B) The rate of DSB repair was also analysed in MEFs defective in DNA ligase IV (LigIV^{-/-}p53^{-/-}) as in (A). Compared with control, the LigIV^{-/-}p53^{-/-} show no detectable rejoining up to 72 h post irradiation (3 Gy). (C) C-HA-XLF^{WT} (XLF), C-HA-XLF^{11insT} (11insT) and empty vector (HA) were transfected into MRC5BIVA cells. XLF was immunoprecipitated from 500 μ g WCE using α -HA antibodies. WCE (50 μ g) and α -HA immunoprecipitates (HA-IP) were analysed with α -HA or α -XLF antibodies. No smaller products were detectable in cells transfected with either XLF or 11insT-XLF. (D) Downregulation of XLF reduced the rate of DSB repair in control but not 2BN (XLF-deficient) cells. 1BRhT (control) and 2BNhT (XLF-deficient) were untreated or treated with siXLF and the rate of loss of γ -H2AX foci enumerated after irradiation (3 Gy). The results represent the mean and SD of three experiments. Cells treated with scrambled siRNA gave identical results to untreated cells.

In contrast, DNA ligase IV was efficiently co-immunoprecipitated using α -MYC antibodies under both salt conditions. XLF also co-immunoprecipitated with XRCC4 in the absence of DNA ligase IV, consistent with previous findings that the interaction is via XRCC4 (15).

These findings show that despite their predicted structural similarity, XLF cannot substitute for XRCC4 in stabilizing DNA ligase IV and that the LX complex represents a functional heterodimer with XLF representing a weaker binding partner.

2BN cells show a 3-fold slower rate of DSB rejoining

Despite the different impacts of XLF and LX on ligation, with LX being essential and XLF providing a modest stimulation, patients with mutations in XLF and DNA ligase IV have overlapping clinical features. To examine loss of XLF function on ds ligation *in vivo*, we monitored DSB rejoining in two XLF-defective cell lines (2BN and F07/402) by enumerating the rate of loss of γ -H2AX foci following exposure to 3 Gy IR in G0/G1 phase primary cells (36). We included two LIG4 cell lines for comparison (Figure 4A). Since DNA ligase IV is essential, all LIG4 cell lines harbour hypomorphic mutations. 180BR is derived from a patient with mild clinical features (no overt immunodeficiency), whilst 495GOS, was derived from a LIG4 syndrome patient with more severe clinical

features of similar severity to those displayed by the 2BN patient. The second XLF defective patient, F07/402, was similar in severity displaying SCID, microcephaly and developmental delay. Both XLF-defective cell lines showed slow but residual DSB rejoining similar to that observed in the LIG4 cell lines. In contrast, a DNA ligase IV null mouse embryo fibroblast (MEF) displayed almost no detectable DSB rejoining when held in G0 phase up to 72 h post irradiation (Figure 4B). Similar results were obtained with the Chinese Hamster Ovary (CHO) cell line, XR-1, which is defective in XRCC4 (Supplementary Figure S3).

The interpretation of these findings requires an evaluation of the impact of the mutational changes. F07/402 harbours a homozygous R57X (C169T) mutation, which is predicted to result in a severely truncated protein. Even a point mutational change at R57, which was observed in another XLF patient and was modelled onto the XLF structure, was predicted to be highly destabilizing (7,18). Thus, it is highly likely that this cell line will not retain residual XLF function. We were unable to examine XLF expression in these poorly growing cells. 2BN cells harbour a homozygous 11insT mutation and have no detectable residual XLF protein (Figure 3B). Even if there was a low level of read through of the termination codon at position 139, the majority of the cDNA would be out of frame and likely non-functional. However, the use of

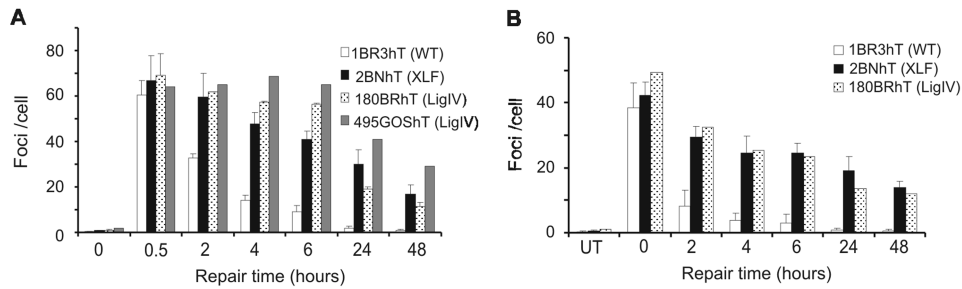


Figure 5. 2BN cells show reduce DSB rejoining after Neocarzinostatin and Etoposide. (A) Control (1BR3hT), XLF-deficient (2BNhT) and LIG4 syndrome (180BRhT and GOShT) cells were analysed for γ -H2AX foci loss following exposure to 50 ng/ml of Neocarzinostatin (NCS). (B) 1BR3hT, 2BNhT and 180BRhT cells were analysed for γ -H2AX foci loss after exposure to 20 μ M of Etoposide.

in-frame translational initiation codons producing a C-terminal fragment could potentially lead to residual activity. Using polyclonal antibodies raised to bacterially expressed full-length human XLF protein (α -XLF), we failed to detect expression of any fragments in 2BN cell extracts of the anticipated size (data not shown). To examine further whether a downstream initiation codon might be utilized, we transfected wild type or mutant C-terminal HA-tagged *XLF* cDNA (C-HA-XLF^{WT} or C-HA-XLF^{11insT}) into MRC5BIVA cells and examined protein expression using α -XLF and α -HA antibodies from cell extracts or following immunoprecipitation using α -HA antibodies. Transfection with C-HA-XLF^{WT} cDNA resulted in expression of XLF detectable using α -XLF and α -HA antibodies, either with or without α -HA immunoprecipitation. No residual XLF protein or any smaller fragments were detectable following transfection with C-HA-XLF^{11insT} cDNA (Figure 4C). Indeed, the only bands present were those observed using an empty vector expressing HA alone. We conclude that there is unlikely to be residual expression of C-terminal XLF fragments in 2BN cells.

To gain further evidence that 2BN cells might lack residual XLF function, we carried out XLF siRNA using 1BRhT cells and observed impaired DSB rejoining albeit less marked than that observed in 2BNhT cells (Figure 4D). Notably, the DSB rejoining in 2BNhT cells subjected to XLF siRNA was unchanged. Together, these findings provide strong evidence that 2BN cells are unlikely to retain significant residual XLF expression and hence function. Since their rate of DSB rejoining is reduced only 3-fold compared to control cells, this strongly suggests that XLF facilitates but is not essential for NHEJ.

2BN cells are similarly impaired in rejoining DSBs induced by Etoposide and Neocarzinostatin

To examine whether XLF might be required for the repair of a specific class of DNA ends, we examined the rate of DSB repair in 2BN following exposure to two additional DNA damaging agents that induce different types of DSBs. Neocarzinostatin produces a relatively homogeneous type of DSB possessing 3' phosphoglycolate and 5' phosphate termini, which lack the complexity of radiation induced DSBs. Etoposide, an inhibitor of topoisomerase

II, also induces a homogenous class of DSB bound by an inactive topoisomerase II molecule. The rate of DSB repair was reduced to a similar magnitude in 2BN cells compared to that observed in LigIV syndrome cell lines suggesting impaired rejoining of multiple classes of DSBs (Figure 5A and B).

DISCUSSION

XLF, a recently identified component of the NHEJ machinery, is structurally related to XRCC4 (18,19) and can promote the ligation step of NHEJ. Two impacts for XLF on ligation have been described; first, the stimulation of ligation has been suggested to occur via an end-bridging mechanism (22,33) and second, XLF has been reported to promote the ligation of mismatched and noncohesive DNA ends (26).

We examined the impact of XLF on LX activities. XLF stimulated LX adenylation activity following de-adenylation by PPI treatment, and also promoted its re-adenylation following ligation, a rate limiting reaction. As a consequence of this recharging, we observed a stimulation of LX-mediated ds ligation under physiological concentrations of ATP. This stimulation did not occur in the presence of an ATP analogue that cannot be hydrolysed to AMP and PPI. Taken together, our findings show that XLF enhances a range of LX activities in the presence or absence of DNA. A fundamental impact on LX activities is consistent with structural studies and demonstrates an effect that does not depend solely on enhanced end-bridging (18,19).

XRCC4 has also been shown to stimulate adenylation in the absence of DNA (37). We have not been able to express sufficient levels of DNA ligase IV alone to examine whether XLF stimulates DNA ligase IV in the absence of XRCC4. However, since our experiments are conducted with LX complexes, the impact of XLF must be additive to any impact of XRCC4.

XRCC4 and DNA ligase IV form a stable complex (35,38). Moreover, DNA ligase IV is poorly expressed *in vivo* and *in vitro* in the absence of XRCC4 and, although less dramatic, XRCC4 expression is reduced *in vivo* in the absence of DNA ligase IV (Figure 3B) (34,38). In contrast, 2BN cells, despite having no detectable XLF protein or residual function, have normal levels of XRCC4 and

DNA ligase IV. Furthermore, XLF is stable in the absence of DNA ligase IV. The interaction of XLF with LX is severely reduced even at moderate ionic strength (≥ 0.3 M NaCl) and, although we were able to co-express XLF, XRCC4 and DNA ligase IV in insect cells, we failed to purify an intact complex with XLF separating from the LX complex even following a mild purification procedure. Collectively, our findings demonstrate that the XLF–XRCC4–DNA ligase IV relationship is not an equal partnership with XLF being less tightly bound to the strongly associated XRCC4–DNA LigIV heterodimer. Moreover, XLF cannot fulfil an important function of XRCC4, namely the stabilization and solubilization of DNA ligase IV. These findings are consistent with the recently described structural studies on XLF, which have revealed a structure that overlaps with, but is distinct, to XRCC4 (18,19). Our findings are also consistent with a study showing that XLF does not impact on LX association or recruitment to DSBs (16).

The clinical features of LIG4 syndrome and XLF-deficient patients are overlapping, which is, perhaps, surprising given that XLF stimulates but is non-essential for LX activity. We compared two XLF-deficient cell lines, F07/402 and 2BN, with two LIG4-deficient lines. Since LIG4 is essential, all LIG4 cell lines harbour hypomorphic mutations with residual protein being detectable, and residual activity, where analysed in detail, ranging from 1% to 10% of control activity (28,39). 180BR cells were derived from a mild LIG4 patient who did not display overt immunodeficiency but showed an over-response to radiotherapy. The cells harbour a homozygous point mutational change (R287H) that reduces LIG4 ds ligation activity (9). 495GOS cells were derived from a more clinically severe patient who required bone marrow transplantation at an early age but expressed detectable residual DNA ligase IV protein. The two XLF patients also had severe immunodeficiency that necessitated bone marrow transplantation (6) (unpublished clinical investigations). 2BN cells retain little, if any, residual function since (i) the mutation causes a highly truncated protein with no evidence of re-initiation, (ii) there is no detectable residual protein and (iii) there is no added impact of XLF siRNA on DSB repair. Whilst LigIV and XRCC4 null cell lines show a near lack of DSB rejoining in G0 phase, the LIG4 and XLF patient cell lines show markedly impaired but residual DSB rejoining. The residual DSB rejoining in LIG4 syndrome cell lines demonstrates the substantial residual DSB rejoining conferred by the hypomorphic mutations. Interestingly, the 180BR cell line, derived from the clinically mild patient, displays a slightly milder DSB repair defect in the γ H2AX assay compared to the more clinically severe 495GOS patient (Figure 4A). The DSB repair defects in both XLF cell lines lie between the two LIG4 syndrome cell lines. Thus, despite the differential impacts of XLF and DNA ligase IV on ligation activity *in vitro*, the patient cell lines display overlapping DSB repair deficiencies, providing an explanation for their overlapping clinical features. Although it is difficult to entirely rule out the impact of low residual XLF activity, these cellular studies, taken together with our biochemical analysis, strongly suggest that, unlike DNA ligase IV and

XRCC4, which are essential for NHEJ, XLF is facilitating but non-essential and enhances DSB rejoining around 3-fold *in vivo*. Recently, Li *et al.* 2008 (40) described an XLF-deficient mouse deleted for exons 4/5. Although it is unclear if this represents a null phenotype, the mouse was viable unlike XRCC4 and DNA ligase IV null mice. Since the essential role of XRCC4 and DNA ligase IV appears to represent NHEJ, this finding is consistent with our notion that XLF has a non-essential role in NHEJ compared to XRCC4 and DNA ligase IV. Interestingly, XLF appears to be dispensable for lymphoid development in mice suggesting that in lymphoid cells, at least, it is not an essential NHEJ component.

On the basis of our findings, we propose a working model whereby LX can be recharged *in situ* following ligation to promote ds ligation by a single DNA ligase IV molecule. The reduced rate of DSB rejoining in the absence of XLF could reflect its occurrence by two independent ligation events. The stoichiometry of NHEJ rejoining proteins is currently unknown. It is likely that a Ku heterodimer binds to a DSB end and recruits one DNA–PKcs subunit per end. However, whether one or two ligase complexes are recruited to carry out the two ligation reactions is currently unclear. The recent structural studies show that the interaction between XRCC4 and XLF occurs through their head domains, but the exact stoichiometry of the complex has not been defined (18,19). Li *et al.* suggested that the most consistent stoichiometry was a 2:2:1 XRCC4–XLF–ligase IV complex but the number of these complexes at a DSB is not clear. Our model would predict a complex which would only contain a single molecule of ligase IV.

XLF has also been shown to promote rejoining of mismatched and non-cohesive ends *in vitro* (26). Our findings are not incompatible with these results, since the ability to recharge LX could enhance end processing linked to ligation. It is important to note, however, that our ligation uses compatible DNA ends. Furthermore, we did not observe any evidence from our *in vivo* analysis for a differential impact of XLF-mediated rejoining of readily ligatable ends generated by Etoposide compared to ends generated by Neocarzinostatin, which are relatively homogeneous and thus should all require or be independent of XLF, nor those generated by radiation, which cover a range of complexities. It is also noteworthy that XLF is required for the rejoining of the signal ends generated during V(D)J recombination *in vivo*, which arise as blunt ds ends (6,7). Our findings are consistent with the ability of XLF to enhance, but not be essential for, all DSB rejoining *in vivo*. It is interesting to note also that, Nej1p, the yeast homologue of XLF, appears to be dispensable for NHEJ at low levels of DSBs, which would be consistent with a non-essential role in promoting re-adenylation (41).

In conclusion, we provide the first demonstration that XLF stimulates LX adenylation activity, and that this can promote ds ligation in the presence of ATP by recharging LX following ligation. We examine two XLF-deficient cell lines, which have little, if any, residual XLF function and show that they have approximately 3-fold slower DSB rejoining activity *in vivo*. We suggest that XLF is

non-essential for NHEJ but facilitates DSB rejoining by enhancing LX adenylation and hence ligation. We suggest a model whereby XLF enhances ds ligation by recharging LX to promote ds ligation by a single DNA ligase IV molecule.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Susan Lees-Miller for the generous gift of DNA-PK.

FUNDING

Medical Research Council; the Association for International Cancer Research; the Department of Health, European Community Integrated project DNA repair (LSHG-CT-2005-512113); European Union RiscRad (FI6R-CT-2003-508842-). Alberta Heritage Foundation for Medical Research to AAG. Funding for open access charge: Medical Research Council; the Association for International Cancer Research; the Department of Health, European Community Integrated project DNA 15 repair (LSHG-CT-2005-512113); European Union RiscRad (FI6R-CT-2003-508842-). Alberta Heritage Foundation for Medical Research to AAG.

Conflict of interest statement. None declared.

REFERENCES

- Weterings,E. and van Gent,D.C. (2004) The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair*, **3**, 1425–1435.
- Jeggo,P.A. (2005) Genome instability in cancer development. *Adv. Exp. Med. Biol.*, **570**, 175–197.
- Gellert,M. (2002) V(D)J recombination: RAG proteins, repair factors, and regulation. *Annu. Rev. Biochem.*, **71**, 101–132.
- O'Driscoll,M., Cersaletti,K.M., Girard,P.-M., Dai,Y., Stumm,M., Kysela,B., Hirsch,B., Gennery,A., Palmer,S.E., Seidel,J. *et al.* (2001) DNA Ligase IV mutations identified in patients exhibiting development delay and immunodeficiency. *Mol. Cell*, **8**, 1175–1185.
- Moshous,D., Callebaut,I., de Chasseval,R., Corneo,B., Cavazzana-Calvo,M., Le Deist,F., Tezcan,I., Sanal,O., Bertrand,Y., Philippe,N. *et al.* (2001) Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency. *Cell*, **105**, 177–186.
- Dai,Y., Kysela,B., Hanakahi,L.A., Manolis,K., Riballo,E., Stumm,M., Harville,T.O., West,S.C., Oettinger,M.A. and Jeggo,P.A. (2003) Non-homologous end joining and V(D)J recombination require an additional factor. *Proc. Natl Acad. Sci. USA*, **100**, 2462–2467.
- Buck,D., Malivert,L., de Chasseval,R., Barraud,A., Fondaneche,M.C., Sanal,O., Plebani,A., Stephan,J.L., Hufnagel,M., le Deist,F. *et al.* (2006) Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*, **124**, 287–299.
- Plowman,P.N., Bridges,B.A., Arlett,C.F., Hinney,A. and Kingston,J.E. (1990) An instance of clinical radiation morbidity and cellular radiosensitivity, not associated with ataxia-telangiectasia. *Br. J. Radiol.*, **63**, 624–628.
- Riballo,E., Critchlow,S.E., Teo,S.H., Doherty,A.J., Priestley,A., Broughton,B., Kysela,B., Beamish,H., Plowman,N., Arlett,C.F. *et al.* (1999) Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. *Curr. Biol.*, **19**, 699–702.
- Walker,J.R., Corpina,R.A. and Goldberg,J. (2001) Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature*, **412**, 607–614.
- Goodarzi,A.A., Yu,Y., Riballo,E., Douglas,P., Walker,S.A., Ye,R., Harer,C., Marchetti,C., Morrice,N., Jeggo,P.A. *et al.* (2006) DNA-PK autophosphorylation facilitates Artemis endonuclease activity. *EMBO J.*, **25**, 3880–3889.
- Meek,K., Douglas,P., Cui,X., Ding,Q. and Lees-Miller,S.P. (2007) Trans autophosphorylation at DNA-dependent protein kinase's two major autophosphorylation site clusters facilitates end processing but not end joining. *Mol. Cell Biol.*, **27**, 3881–3890.
- Nick McElhinny,S.A., Snowden,C.M., McCarville,J. and Ramsden,D.A. (2000) Ku recruits the XRCC4-ligase IV complex to DNA ends. *Mol. Cell Biol.*, **20**, 2996–3003.
- Calsou,P., Delteil,C., Frit,P., Drouet,J. and Salles,B. (2003) Coordinated assembly of Ku and p460 subunits of the DNA-dependent protein kinase on DNA ends is necessary for XRCC4-ligase IV recruitment. *J. Mol. Biol.*, **326**, 93–103.
- Ahnesorg,P., Smith,P. and Jackson,S.P. (2006) XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell*, **124**, 301–313.
- Wu,P.Y., Frit,P., Malivert,L., Revy,P., Biard,D., Salles,B. and Calsou,P. (2007) Interplay between Cernunnos-XLF and nonhomologous end-joining proteins at DNA ends in the cell. *J. Biol. Chem.*, **282**, 31937–31943.
- Yano,K., Morotomi-Yano,K., Wang,S.Y., Uematsu,N., Lee,K.J., Asaithamby,A., Weterings,E. and Chen,D.J. (2008) Ku recruits XLF to DNA double-strand breaks. *EMBO Rep.*, **9**, 91–96.
- Li,Y., Chirgadze,D.Y., Bolanos-Garcia,V.M., Sibanda,B.L., Davies,O.R., Ahnesorg,P., Jackson,S.P. and Blundell,T.L. (2007) Crystal structure of human XLF/Cernunnos reveals unexpected differences from XRCC4 with implications for NHEJ. *EMBO J.*, **27**, 290–300.
- Andres,S.N., Modesti,M., Tsai,C.J., Chu,G. and Junop,M.S. (2007) Crystal structure of human XLF: a twist in nonhomologous DNA end-joining. *Mol. Cell*, **28**, 1093–1101.
- Callebaut,I., Malivert,L., Fischer,A., Mornon,J.P., Revy,P. and de Villartay,J.P. (2006) Cernunnos interacts with the XRCC4 x DNA-ligase IV complex and is homologous to the yeast nonhomologous end-joining factor Nej1. *J. Biol. Chem.*, **281**, 13857–13860.
- Gu,J., Lu,H., Tippin,B., Shimazaki,N., Goodman,M.F. and Lieber,M.R. (2007) XRCC4:DNA ligase IV can ligate incompatible DNA ends and can ligate across gaps. *EMBO J.*, **26**, 1010–1023.
- Hentges,P., Ahnesorg,P., Pitcher,R.S., Bruce,C.K., Kysela,B., Green,A.J., Bianchi,J., Wilson,T.E., Jackson,S.P. and Doherty,A.J. (2006) Evolutionary and functional conservation of the DNA nonhomologous end-joining protein, XLF/Cernunnos. *J. Biol. Chem.*, **281**, 37517–37526.
- Nagaki,S., Yamamoto,M., Yumoto,Y., Shirakawa,H., Yoshida,M. and Teraoka,H. (1998) Non-histone chromosomal proteins HMG1 and 2 enhance ligation reaction of DNA double-strand breaks. *Biochem. Biophys. Res. Commun.*, **246**, 137–141.
- Stiff,T., Shtivelman,E., Jeggo,P. and Kysela,B. (2004) AHNK interacts with the DNA ligase IV-XRCC4 complex and stimulates DNA ligase IV mediated double stranded ligation. *DNA Repair*, **3**, 245–256.
- Ramsden,D.A. and Gellert,M. (1998) Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. *EMBO J.*, **17**, 609–614.
- Tsai,C.J., Kim,S.A. and Chu,G. (2007) Cernunnos/XLF promotes the ligation of mismatched and noncohesive DNA ends. *Proc. Natl Acad. Sci. USA*, **104**, 7851–7856.
- Wang,Y., Lamarche,B.J. and Tsai,M.D. (2007) Human DNA ligase IV and the ligase IV/XRCC4 complex: analysis of nick ligation fidelity. *Biochemistry*, **46**, 4962–4976.
- Riballo,E., Doherty,A.J., Dai,Y., Stiff,T., Oettinger,M.A., Jeggo,P.A. and Kysela,B. (2001) Cellular and biochemical impact of a mutation in DNA ligase IV conferring clinical radiosensitivity. *J. Biol. Chem.*, **276**, 31124–31132.

29. Arlett, C.F. and Cole, J. (1988) The role of mammalian cell mutation assays in mutagenicity and carcinogenicity testing. *Mutagenesis*, **3**, 455–458.
30. Rothkamm, K. and Lobrich, M. (2003) Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc. Natl Acad. Sci. USA*, **100**, 5057–5062.
31. Marchetti, C., Walker, S.A., Odreman, F., Vindigni, A., Doherty, A.J. and Jeggo, P. (2006) Identification of a novel motif in DNA ligases exemplified by DNA ligase IV. *DNA Repair*, **5**, 788–798.
32. Merkle, D., Douglas, P., Moorhead, G.B., Leonenko, Z., Yu, Y., Cramb, D., Bazett-Jones, D.P. and Lees-Miller, S.P. (2002) The DNA-dependent protein kinase interacts with DNA to form a protein-DNA complex that is disrupted by phosphorylation. *Biochemistry*, **41**, 12706–12714.
33. Lu, H., Pannicke, U., Schwarz, K. and Lieber, M.R. (2007) Length-dependent binding of human XLF to DNA and stimulation of XRCC4.DNA ligase IV activity. *J. Biol. Chem.*, **282**, 11155–11162.
34. Bryans, M., Valenzano, M.C. and Stamato, T.D. (1999) Absence of DNA ligase IV protein in XR-1 cells: evidence for stabilization by XRCC4. *Mutat. Res.*, **433**, 53–58.
35. Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T.E., Mann, M. and Lieber, M.R. (1997) Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature*, **388**, 492–495.
36. Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G.C., Recio, M.J., Reis, C., Dahm, K., Fricke, A., Krempler, A. *et al.* (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol. Cell*, **16**, 715–724.
37. Modesti, M., Hesse, J.E. and Gellert, M. (1999) DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity. *EMBO J.*, **18**, 2008–2018.
38. Critchlow, S.E., Bowater, R.P. and Jackson, S.P. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Curr. Biol.*, **7**, 588–598.
39. Girard, P.-M., Kysela, B., Harer, C.J., Doherty, A.J. and Jeggo, P.A. (2004) Analysis of DNA ligase IV mutations found in LIG4 syndrome patients: the impact of two linked polymorphisms. *Hum. Mol. Gene.*, **13**, 2369–2376.
40. Li, G., Alt, F.W., Cheng, H.L., Brush, J.W., Goff, P.H., Murphy, M.M., Franco, S., Zhang, Y. and Zha, S. (2008) Lymphocyte-specific compensation for XLF/cernunnos end-joining functions in V(D)J recombination. *Mol. Cell*, **31**, 631–640.
41. Ahnesorg, P. and Jackson, S.P. (2007) The non-homologous end-joining protein Nej1p is a target of the DNA damage checkpoint. *DNA Repair*, **6**, 190–201.