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

Article (Accepted Version)

XLF-Cernunnos promotes DNA ligase IV-XRCC4 readenylation following ligation.

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

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

Character Count: 51,520.

Corresponding Author: P. A. Jeggo p.a.jeggo@sussex.ac.uk
0044 1273 678482
0044 1273 678121

Running title: XLF promotes LX readenylation following ligation.

Keywords: DNA repair, double strand break repair, DNA ligation, radiosensitivity, DNA non homologous end-joining.

Abstract.

XLF-Cernunnos (XLF) is a recently identified co-factor of the DNA Ligase IV/XRCC4 (LX) complex, which functions in DNA non-homologous end joining (NHEJ). XLF has been reported to promote LX double stranded (ds) ligation activity by end-bridging. We show that XLF is a weaker binding partner of the tightly associated LX complex and, unlike XRCC4, is dispensable for LX stability. XLF enhances LX adenylation and strikingly promotes a three fold increase in the rate of LX re-adenylation following de-adenylation by ligation, a reaction which is otherwise rate limiting. Whilst ATP alone fails to stimulate LX ds ligation activity, consistent with readenylation being rate limiting for ligation, addition of XLF and ATP promotes ligation. We also observe stimulation of LX activity by XLF in the absence of ATP, potentially attributable to end-bridging. Cellular analysis of 2BN cells, which do not express detectable XLF protein, show that they have a 3 fold decrease in DSB rejoining supporting our biochemical findings that XLF enhances NHEJ by promoting recycling of LX following ligation.

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 components of the NHEJ machinery are exquisitely radiosensitive and DSB repair defective (Jeggo, 2005; Weterings and van Gent, 2004). NHEJ also functions to effect rearrangements at site specific DSBs introduced during the process of V(D)J recombination (Bassing and Alt, 2004; Gellert, 2002). Consequently, viable mice deficient in 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, leading to the classification of radiosensitive-(RS)-SCID (Buck et al., 2006; Dai et al., 2003; Moshous et al., 2001; O'Driscoll et al., 2001). One patient received radiotherapy and dramatically over-responded to treatment demonstrating clinical radiosensitivity (Plowman et al., 1990; Riballo et al., 1999).

The first step of NHEJ is binding of the heterodimeric Ku protein to double stranded (ds) DNA ends. The crystal structure of Ku has shown it to be a basket shaped structure with a central core surrounded by a base, a “handle” and two “pillars” (Walker et al., 2001). The central core is of sufficient diameter to allow the threading of Ku onto ds DNA and its translocation along the DNA. Once bound to DNA, Ku recruits the DNA-PK catalytic subunit (DNA-PKcs), creating the active DNA-PK complex (Dvir et al., 1992; Gottlieb and Jackson, 1993). Increasing evidence suggests that DNA-PK undergoes autophosphorylation, which serves to regulate the process and/or facilitate the recruitment of additional proteins required for end processing (Kurimasa et al., 1999). The assembled DNA-PK complex facilitates the recruitment of a ligation complex encompassing XRCC4 and DNA Ligase IV (Calsou et al., 2003; Nick McElhinny et al., 2000).

XLF-Cernunnos, hereafter called XLF, was identified via the analysis of one class of RS-SCID patients with features closely resembling those of LIG4 syndrome patients, a disorder caused by mutations in DNA Ligase IV (Ahnesorg et al., 2006; Buck et al., 2006; Dai et al., 2003). This strongly suggested that XLF is a component of the NHEJ

machinery, which is substantiated by the finding that XLF interacts with XRCC4 (Ahnesorg et al., 2006). Interestingly, XLF is predicted to have a structure similar to XRCC4 with an N-terminal globular head domain and C-terminal coiled coil structure (Ahnesorg et al., 2006). Subsequent analysis revealed that XLF, in fact, represents the mammalian homologue of the yeast Nej1p protein, a factor regulating NHEJ in yeast, and that both proteins belong to an extended XRCC4 family (Callebaut et al., 2006). Since both *LIG4* and *XLF* deficient cell lines are proficient in Ku end-binding activity and display normal DNA-PK activity, the available evidence suggested that XLF is a further factor required for the ligation step of NHEJ (Ahnesorg et al., 2006; Dai et al., 2003). The co-immunoprecipitation of XLF with LX has lead to the suggestion of a tripartite XLF-XRCC4-LigaseIV complex. Further studies have shown that XLF can stimulate LX ligation by promoting end-bridging (Ahnesorg et al., 2006; Gu et al., 2007; Hentges et al., 2006). Since multiple proteins are able to promote end-bridging *in vitro* including DNA-PKcs and Ku, and since DNA ends might be tethered to some extent by the higher order structure of DNA, we considered it unlikely that this would represent the marked *in vivo* role for XLF (Nagaki et al., 1998; Pil et al., 1993; Ramsden and Gellert, 1998; Stiff et al., 2004) and therefore sought further evidence for the impact of XLF on LX activities.

The ligation reaction involves an initial charging step involving the formation of a DNA Ligase IV-adenylate complex involving the catalytic lysine in DNA Ligase IV. LX complexes are expressed in baculovirus, bacteria and human cells as an adenylate complex. The ligation reaction involves transfer of the AMP moiety to the 5' phosphate on the DNA creating a DNA-adenylate complex and releasing uncharged DNA Ligase IV, which needs to be re-adenylated for subsequent ligation activity. A recent study characterising 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 a rate limiting step *in vitro* (Wang et al., 2007). In support of this, we and others have shown that the presence of ATP does not stimulate ds ligation suggesting that recharging of DNA Ligase IV does not occur efficiently. Here, we use biochemical and cellular approaches to probe the role of XLF in NHEJ. Analysis of an *XLF* deficient cell line, 2BN, demonstrated that XLF is dispensable for Ligase IV stability and is a weaker binding partner of the LX complex. We show that XLF promotes a three-fold stimulation

of LX re-adenylation following ligation. Consistent with these findings, we show that XLF stimulates LX activity in the presence of ATP. Examination of XLF deficient 2BN cells provides an explanation for the overlapping clinical features of XLF and DNA ligase IV deficient patients. Collectively, our data suggest that XLF is facilitating but non-essential for DSB repair by promoting recharging of LX following ligation, an activity that will benefit ds ligation.

Results.

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

To gain insight into XLF function *in vivo*, we used the 2BN cell line, which was derived from a severe combined immunodeficiency (SCID) patient with a single base pair (+T) insertion at position 11 (11insT) in the first coding exon of the *XLF* gene. The insertion creates a termination codon at position 139 (Ahnesorg et al., 2006; Dai et al., 2003). Using polyclonal antibodies raised to bacterially expressed, full length, human XLF protein (α-XLF), we were unable to detect any residual XLF protein in 2BN cell extracts by immunofluorescence or immunoblotting (Figures 1A and B) despite a strong signal obtained by immunoblotting control cell extracts consolidating previous analysis of 2BN cells using a distinct α-XLF antibody (Ahnesorg et al., 2006). We were also unable to detect any smaller fragments in the immunoblots in 2BN cells providing no evidence that translational reinitiation occurs in 2BN cells (results not shown). Given the lack of any significant expression of XLF in 2BN cells, we examined the interdependence of XLF, XRCC4 and DNA Ligase IV expression and protein stability. Western blotting and immunofluorescence analysis showed that XRCC4 and DNA Ligase IV are expressed normally and localise to the nucleus in 2BN cells (Figures 1A and B). In contrast, XR-1 cells, which lack XRCC4, express low levels of DNA Ligase IV, as reported previously (Figure 1B) (Bryans et al., 1999). Moreover, XRCC4 expression in a *LIG4* null cell line, N114P2, is approximately one third reduced compared to the parent line, Nalm6 (Figure 1B). XLF was expressed normally in DNA Ligase IV-defective N114P2 cells. The expression of XLF in the absence of XRCC4 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 localisation.

Since 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 (N-FLAG-

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 (Figure1C). This contrasts with the strong interaction between XRCC4 and DNA Ligase IV which is stable even at 1 M NaCl (Grawunder et al., 1997). 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 1D). Immunoprecipitation using α-MYC antibodies efficiently co-immunoprecipitated XLF at 0.12 mM NaCl but not at 1 M NaCl. In contrast, DNA Ligase IV was efficiently co-immunoprecipitated using α-MYC antibodies under physiological and high 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 (Ahnesorg et al., 2006).

Altogether, these data show that, whereas XRCC4 tightly interacts with DNA Ligase IV enhancing its stable expression, the interaction between LX and XLF is considerably weaker. Thus, despite their predicted structural similarity, XLF cannot substitute for XRCC4 in stabilising DNA Ligase IV. These findings show that the LX complex represents a functional heterodimer with XLF representing a weaker binding partner.

XLF stimulates Ligase IV re-adenylation following ligation.

XLF has been reported to promote LX ligation activity by end-bridging (Ahnesorg et al., 2006; Gu et al., 2007; Hentges et al., 2006). However, since multiple proteins stimulate LX activity by end-bridging, including Ku, DNA-PKcs, HMG proteins and AHNAK, we examined further the impact of XLF on LX functions. The first step of the ligation reaction is binding of AMP to the active site lysine of DNA Ligase IV. We examined whether XLF might affect LX adenylation activity. Since we were unable to purify a co-expressed trimeric complex due to the weak association of XLF with LX at greater than physiological salt, we used separately purified, bacterially expressed XLF and insect cell expressed LX. We considered this reasonable given the nature of the interactions described above. LX is expressed in baculovirus as a pre-adenylate complex and assessment of adenylation activity is normally carried out following de-adenylation using

high concentrations of inorganic pyrophosphate (PPi) which causes release of the AMP moiety. Using 5 pmols of XLF and 1 pmol of LX, we observed a 2-3 fold stimulation in the overall rate of adenylation over the course of 20 minutes (Figure 2A). A similar degree of stimulation was observed using between 5 and 30 pmols XLF.

We and others have previously been unable to observe re-adenylation of LX following ligation, consistent with the inability of ATP to stimulate LX in ligation assays (Riballo et al., 2001; Wang et al., 2007). Therefore, we considered the possibility that XLF might play a role in recharging LX following ligation. To investigate this, we first examined the impact of ligatable DNA on the adenylation reaction using LX complexes that had been previously de-adenylated following PPi treatment. We utilised a 445bp pBluescript fragment that we have previously used in ds ligation reactions (Riballo et al., 2001) and observed that the presence of DNA decreases adenylate complex formation regardless of the presence of XLF (Figure 2B). This suppressive impact was more marked at later times, consistent with the possibility that the act of ligation causes de-adenylation of LX thereby reducing the overall amount of adenylate complex observed.

To determine whether XLF might stimulate re-adenylation following ligation, we adapted our assay to examine adenylate complex formation without prior de-charging of LX complexes by PPi treatment. In fact, we examined whether ligation was able to de-charge the LX complexes allowing subsequent re-adenylation. To achieve this, we allowed ligation to take place in the absence of ATP, with or without XLF during a 15 minute pre-incubation period, prior to the addition of radiolabelled ATP used to monitor adenylate complex formation (Figure 2C). Strikingly, we observed that XLF promoted LX adenylation following incubation in the presence of DNA, while no such activity was observed in the absence of XLF. This provides the first demonstration that re-adenylation of LX can occur following ligation. To verify that ligation had taken place during the reaction above, we monitored ligation during the pre-incubation phase in the absence of ATP and subsequently when ATP (with or without XLF) was added (Figure 2D). We could observe ligation products during the pre-incubation reaction consistent with the notion that de-adenylation occurred as a consequence of ligation during this pre-incubation step. However, ligation reached a maximum at 15 min and further stimulation following the 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 the LX complexes become recharged during the reaction since optimisation of our ability to detect LX-^{32}P-adenylate complex formation required using radiolabelled ATP without any added cold ATP. Thus, the ATP concentrations are likely below the optimal Km for ATP for DNA Ligase IV. The presence of XLF during the 15 minutes pre-incubation step did not increase either the rate of LX adenylation (Figure 2C) 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 utilised, 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 2E). Thus, 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.

Finally, we aimed to determine whether XLF was able to stimulate LX adenylation using physiologically relevant ratios of XLF and LX. To achieve this, we used α-XRCC4 antibodies to immunoprecipitate LX-XLF complexes from whole cell extracts using 0.12 M NaCl and employed a high salt (1M NaCl) wash to discharge XLF (Figure 1C). De-adenylation of the complexes was carried out by PPi treatment or by incubation with DNA. XLF stimulated LX adenylation activity following PPi treatment (+PPi, 1M NaCl versus +PPi, 0.12M NaCl) but strikingly, stimulation was even greater when DNA was used to de-charge the LX complexes (+DNA, 1 M NaCl versus + DNA, 0.12M NaCl). Thus, even though our biochemical studies required 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 able to stimulate LX re-adenylation linked to ligation.

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

Previous studies have shown that XLF stimulates LX ds ligation activity (Gu et al., 2007; Hentges et al., 2006). Our finding that XLF stimulates recharging of LX 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 2D), this was potentially attributable to the low ATP concentrations utilised. We, therefore, examined the impact of physiological ATP concentrations on the ability of XLF to stimulate LX ds ligation. Firstly, we titrated the amount of XLF stimulating 125 fmoles LX, which effects only a low level of ligation in our assay (Figure 3A). We observed a broad peak of stimulation from 2.5-30 pmol XLF, which varied slightly with individual LX preparations. We next examined the ability of 17 pmols XLF to stimulate ligation effected by differing amounts of LX in the presence and absence of ATP (Figure 3B). In the absence of XLF, little ds ligation was observed. In agreement with previous findings and the discussion above, addition of ATP did not stimulate LX activity consistent with the notion that LX is not subject to re-adenylation following ligation (Figure 3B, compare left and right panels) (Riballo et al., 2001; Wang et al., 2007). XLF provided some stimulation in the absence of ATP but further stimulation was observed in the presence of ATP (Figure 3B right panel). Significantly, when very high amounts of LX were employed (50 fmols), XLF provided no further stimulatory effect.

It has been suggested that XLF might function as a bridging factor to facilitate the alignment and rejoining of ds DNA ends (Hentges et al., 2006; Lu et al., 2007). 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 together with XLF and an equal amount of an identical, ^{32}P-end labelled substrate. Intermolecular association of the two substrates was assessed by monitoring the ability of the streptavidin beads to retain the ^{32}P-labelled duplex following multiple wash steps (Figure 3C). XLF significantly enhanced the recovery of radio-labelled DNA associated with beads demonstrating that it facilitates DNA end-bridging. As a control, we carried out the reaction using purified DNA-PK, which also promotes end-bridging (Cary et al., 1997; Merkle et al., 2002). Mol for mol, DNA-PK had a greater level of duplex-bridging activity compared to XLF and, importantly, the addition of XLF together with DNA-PK was not additive. These findings are consistent with the notion that XLF promotion of

ligation in the absence of ATP might be attributable to its ability to promote end-bridging. It is noteworthy that the experiments carried out in Figure 2D used a lower XLF to LX molar ratio (~5:1), which was optimal for observing the stimulatory effect of XLF on LX adenylation. In contrast, the ligation experiments in Figure 3B required a higher molar ratio of XLF to LX (~75:1). Since the ratio of LX to DNA ends remains essentially the same between Figures 2D and 3B (~5:1 versus ~2:1, respectively), it is possible that the higher level of XLF is required to promote sufficient DNA end bridging to observe and effect. Since multiple proteins promote end-bridging and stimulate *in vitro* ligation reactions, it is currently unclear whether this represents a significant role for XLF under physiological conditions although it may provide an additional impact on ligation.

The ATP-dependent impact of XLF is more unique, however, and consistent with the notion that XLF promotes recharging of LX. To further examine this, we also looked at the impact of XLF on the kinetics and maximal level of ligation with or without ATP (Figure 3D). In the absence of ATP, stimulation of ligation by XLF occurred within the first 3 minutes and little further stimulation was observed. Addition of ATP in the presence of XLF did not further enhance the initial rate of the ds ligation but dramatically enhanced the maximal level obtained. Taken together, 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.

2BN cells show a three fold slower rate of DSB rejoining.

Despite the very 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 gain *in vivo* evidence for the impact of the loss of XLF function on ds ligation, we examined DSB rejoining in 2BN cells. Our findings in Figure 1A and B demonstrate that 2BN cells do not express detectable full length XLF protein, consistent with the 1 bp insertion in the cDNA. Even if there was a low level of read through of the termination codon generated at position 139, the majority of the cDNA would be out of frame and likely non-functional. However, one route that could potentially lead to residual activity is the possible use of in frame translational initiation

codons producing a C-terminal fragment. Using polyclonal antibodies raised to bacterially-expressed full length human XLF protein (α-XLF), we did not detect expression of a smaller fragment in 2BN cell extracts of the anticipated size (data not shown). To examine further whether a downstream initiation codon might be utilised, we transfected wild type or mutant C-terminal HA-tagged *XLF* cDNA (C-HA-XLF^{WT} or C-HA-XLF^{11insT}) into MRC5VIBA 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 4A). Indeed, the only bands present were those observed using an empty vector expressing HA alone. We conclude that there is unlikely to be significant expression of C-terminal XLF fragments in 2BN cells.

Taken together, these findings provide strong evidence that 2BN cells are unlikely to retain any significant residual XLF expression and hence function. We, therefore, monitored DSB rejoining in 2BN cells to examine the impact of marked loss of XLF function *in vivo*. We enumerated the rate of loss of γ-H2AX foci following exposure to 3 Gy IR in G0/G1 phase primary cells, which we have shown previously represents a highly sensitive to assay to monitor DSB repair *in vivo* (Riballo et al., 2004). We also included two LIG4 cell lines in our analysis for comparison (Figure 4B). Since DNA ligase IV is essential, all mutation changes in LIG4 cell lines are hypomorphic. One cell line (180BR) was derived from a patient with mild clinical features (no overt immunodeficiency), whilst the second line (495GOS) has clinical features of similar severity to the 2BN patient. 2BN cells showed slow but residual DSB rejoining that overlaps with that observed in the LIG4 cell lines. In contrast, a DNA Ligase IV null mouse embryo fibroblast (MEF) displayed almost no detectable DSB rejoining up to 72 h post irradiation using the same assay (Figure 4C). To examine the kinetics of rejoining in 2BN cells, we examine additional time points and observed an approximately 3 fold reduced rate of rejoining (Figure 4D). These findings suggest that 2BN cells display low

but residual DSB rejoining activity in G0/G1 phase, consistent with our *in vitro* findings that XLF enhances but is not essential for LX activity.

Discussion.

XLF, a recently identified component of the NHEJ machinery, is predicted to be structurally related to XRCC4 and likely represents the mammalian homologue of Nej1p, a protein reported to regulate NHEJ in diploid yeast (Ahnesorg et al., 2006; Buck et al., 2006; Callebaut et al., 2006; Frank-Vaillant and Marcand, 2001; Hentges et al., 2006; Valencia et al., 2001). XLF interacts with LX and can promote LX ds ligation activity, leading to the suggestion that it represents a third partner of the LX complex (Hentges et al., 2006; Lu et al., 2007). However, it is currently unclear how XLF promotes ds ligation by LX and its precise role in NHEJ. Here, we exploit biochemical and cellular approaches to provide insight into the role of XLF in DSB rejoining by NHEJ.

XRCC4 and DNA Ligase IV form a highly stable complex (Critchlow et al., 1997; Grawunder et al., 1997); our unpublished observations). Moreover, DNA Ligase IV is poorly expressed *in vivo* and *in vitro* in the absence of XRCC4 (Bryans et al., 1999; Critchlow et al., 1997). Conversely, although the impact is less dramatic, XRCC4 has reduced expression *in vivo* in the absence of DNA Ligase IV (Figure 1B). In contrast, 2BN cells, which have no detectable XLF protein or residual function, have normal levels of XRCC4 and DNA Ligase IV, and *vice versa*, XLF is stable in the absence of DNA Ligase IV. Furthermore, the interaction of XLF with the LX complex is severely reduced even at moderately high ionic strength (≥ 0.3 M NaCl). Indeed, although we were able to co-express XLF, XRCC4 and DNA Ligase IV in insect cells, we failed to purify an intact complex since XLF separated from the LX complex using relatively mild purification procedures. Taken together, our findings demonstrate that the XLF-XRCC4-DNA Ligase IV relationship is not an equal partnership with XLF being a weaker binding partner of the tightly complexed XRCC4-DNA LigIV heterodimer. Moreover, XLF cannot fulfil one of the important functions of XRCC4, namely the stabilisation and solubilisation of DNA Ligase IV.

To gain insight into the mechanism underlying the ability of XLF to stimulate LX ds ligation, we examined its impact on LX activities. XLF stimulated LX adenylation

activity following de-adenylation by PPi treatment, and importantly promoted re-adenylation of LX following ligation. Among the DNA ligases, DNA ligase IV is unique in being poorly recharged following ligation (Wang et al., 2007). Our findings present the first demonstration of conditions promoting recharging of LX linked to ligation. We also examined the impact of XLF and ATP on ligation. XLF stimulated LX ligation in the absence of ATP, very likely attributable to its ability to promote end-bridging but it is unclear if this represents a bona fide physiological role. However, the stimulation of LX ds ligation by XLF in the presence of ATP is entirely consistent with an impact on recharging following ligation and importantly shows for the first time that recharging of LX can promote additional ligation. Interestingly, the DNA end binding ability of XLF that underlies its DNA bridging ability may help to retain LX on the DNA end following ligation. Thus, XLF may serve to retain and recharge LX at a ds DNA end to promote concerted ligation of two DNA ends. Such *in situ* recharging would be particularly beneficial for ds ligation.

The overlapping clinical features of LIG4 syndrome and XLF-deficient patients were perhaps surprising given the fact that XLF stimulates but is non-essential for LX activity. We, therefore, examined the impact of marked loss of XLF function *in vivo* by examining the 2BN cell line. The clinical features observed in LIG4 syndrome patients, which include immunodeficiency, microcephaly and developmental delay, cover a range of severities (O'Driscoll et al., 2001; Riballo et al., 1999; van der Burg et al., 2006). One patient (180BR) did not display overt immunodeficiency but was identified following the dramatic over response to radiotherapy. We included analysis of the 180BR cell line as well as that derived from another LIG4 patient (495GOS) who displayed marked immunodeficiency and required bone marrow transplantation at an early age. The 2BN patient also had relatively severe immunodeficiency that necessitated bone marrow transplantion (Dai et al., 2003). Where analysed in detail, the residual ligation activity detected in mutant proteins from LIG4 syndrome patients ranges from 1 to 10%, and residual DNA ligase IV protein is detectable in many LIG4 cell lines (Girard et al., 2004; Riballo et al., 2001). The analysis of DSB rejoining in 2BN cells demonstrates that, in contrast to the near lack of DSB rejoining in a *LigIV* null cell line, there is impaired but residual DSB rejoining. The residual DSB rejoining in LIG4 syndrome cell lines

compared to the defect in the *LigIV* null line demonstrates the impact of residual activity in the LIG4 patients. Thus, the hypomorphic mutations in LIG4 syndrome patients allows substantial residual DSB rejoining. Interestingly, cells from the 180BR patient display a slightly milder defect in DSB rejoining in the γH2AX assay compared to the cell line from the more clinically severe, 495GOS patient (Figre 4B). The DSB repair defect in 2BN cells lies between that observed in the two LIG4 syndrome cell lines. Thus, despite the different impacts of the two proteins on ligation activity *in vitro,* the patient cell lines display overlapping DSB repair deficiencies, providing an explanation for the overlapping clinical features of the patients. Furthermore, although it is difficult to entirely rule out the impact of a low level of residual XLF activity, our results with 2BN cells are consistent with our biochemical findings, namely that XLF facilitates ds ligation by promoting recharging of LX to enhance co-ordinated ds ligation rather than relying on two independent ligation events. Hence, it enhances DSB rejoining around 3 fold.

In conclusion, our findings show that XRCC4 and DNA Ligase IV interact tightly as an interdependent heterodimer whereas XLF is a less tightly associated binding partner. Stable expression of XRCC4 and DNA Ligase IV *in vivo* is interdependent whereas expression of XLF is not influenced by expression of XRCC4 or DNA Ligase IV. XLF stimulates LX ligation activity in an ATP dependent and independent manner. The ATP-independent stimulation of LX could be a consequence of its end-bridging activity but the physiological relevance of this is questionable. We provide evidence that the ATP-dependent stimulation of LX activity is due to the ability of XLF to promote LX adenylation activity following ligation, a reaction that proceeds slowly in the absence of XLF. These findings are consistent with our analysis of 2BN cells, which have very low, if any, residual XLF function and show approximately 3 fold slower DSB rejoining activity *in vivo.* Taken together our findings strongly suggest that the mechanism by which XLF promotes LX ligation is by recharging the enzyme following ligation.

Materials and Methods.

Cell culture.

Primary fibroblasts, 1BR3 (control) and 2BN (XLF-deficient), hTERT immortalised fibroblasts, 48BRhTERT (control) and 2BNhTERT, and SV-40 transformed human fibroblasts, MRC5BiVA, were grown in minimal essential medium (MEM) as described previously (Arlett and Cole, 1988; Dai et al., 2003). 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) 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 11insT mutation into *XLF* cDNA, QuickChange^{R} XL Site-Directed Mutagenesis kit from Stratagene was utilized (Cambridge, UK). MRC5BiVA were transfected using Fugene (Roche, Burgess Hill, UK).

Immunofluorescence and immunoblotting.

Immunofluorescence analysis was carried out as previously described (Rothkamn and Löbrich, 2003). Whole cell extracts for immunobltting and immunoprecipitations were prepared as described previously (Ahnesorg et al., 2006). 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* (Riballo et al., 2001), 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 (Hentges et al., 2006; Marchetti et al., 2006). For XLF purification, the bacterial pellet was lysed in 50 mM Tris pH8, 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 (Merkle et al., 2002).

Adenylation and ligations.

Adenylation and ligations were performed as described previously (Marchetti et al., 2006; Riballo et al., 2001).

Bridging assay for the association of two DNA molecules.

This assay was performed as described previously (Ramsden and Gellert, 1998; Stiff et al., 2004). 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).

Acknowledgements.

We thank Susan Lees-Miller for the generous gift of DNA-PK. The PAJ laboratory is supported by the Medical Research Council, the Association for Internation Cancer Research, the Leukaemia Research Fund, the Department of Health, EU grant (FIGH-CT-200200207) (DNA repair) and EU gramt FI6R-CT-2003-508842 (RiscRad). AAG is funded by the Alberta Heritage Foundation for Medical Research.

References.

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.

Arlett, C.F. and Cole, J. (1988) The role of mammalian cell mutation assays in mutagenicity and carcinogenicity testing. *Mutagenesis*, 3, 455-458.

Bassing, C.H. and Alt, F.W. (2004) The cellular response to general and programmed DNA double strand breaks. *DNA Repair (Amst)*, 3, 781-796.

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.

Buck, D., Malivert, L., de Chasseval, R., Barraud, A., Fondaneche, M.C., Sanal, O., Plebani, A., Stephan, J.L., Hufnagel, M., le Deist, F., Fischer, A., Durandy, A., de Villartay, J.P. and Revy, P. (2006) Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell*, 124, 287-299.

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.

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.

Cary, R.B., Peterson, S.R., Wang, J., Bear, D.G., Bradbury, E.M. and Chen, D.J. (1997) DNA looping by Ku and the DNA-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 4267-4272.

Critchlow, S.E., Bowater, R.P. and Jackson, S.P. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Current Biology*, 7, 588-598.

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. U S A*, 100, 2462-2467.

Dvir, A., Peterson, S.R., Knuth, M.W., Lu, H. and Dynan, W.S. (1992) Ku autoantigen is the regulatory component of a template-associated protein kinase that phosphorylates RNA polymerase II. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 11920-11924.

Frank-Vaillant, M. and Marcand, S. (2001) NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the Ligase IV pathway. *Genes Dev*, 15, 3005-3012.

Gellert, M. (2002) V(D)J recombination: RAG proteins, repair factors, and regulation. *Annu Rev Biochem*, 71, 101-132.

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. *Human Molecular Genetics*, 13, 2369-2376.

Gottlieb, T.M. and Jackson, S.P. (1993) The DNA-dependent protein kinase: requirement of DNA ends and association with Ku Antigen. *Cell*, 72, 131-142.

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.

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 non homologous end joining protein, XLF/Cernunnos. *J Biol Chem*.

Jeggo, P.A. (2005) Genomic Instability in cancer development. In Nigg, E.A. (ed.), *Genome Instability*, Netherlands, pp. 175-197.

Kurimasa, A., Kumano, S., Boubnov, N., Story, M.D., Tung, C., Peterson, S.R. and Chen, D.J. (1999) Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining. *Molecular and Cellular Biology*, 19, 3877-3884.

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.

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 (Amst)*, 5, 788-798.

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.

Moshous, D., Callebaut, I., de Chasseval, R., Corneo, B., Cavazzana-Calvo, M., Le Deist, F., Tezcan, I., Sanal, O., Bertrand, Y., Philippe, N., Fischer, A. and de Villartay, J.P. (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.

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.

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.

O'Driscoll, M., Cerosaletti, K.M., Girard, P.-M., Dai, Y., Stumm, M., Kysela, B., Hirsch, B., Gennery, A., Palmer, S.E., Seidel, J., Gatti, R.A., Varon, R., Oettinger, M.A., Sperling, K., Jeggo, P.A. and Concannon, P. (2001) DNA Ligase IV mutations identified in patients exhibiting development delay and immunodeficiency. *Mol Cell*, 8, 1175-1185.

Pil, P.M., Chow, C.S. and Lippard, S.J. (1993) High-mobility-group 1 protein mediates DNA bending as determined by ring closures. *Proc Natl Acad Sci U S A*, 90, 9465-9469.

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. *British Journal of Radiology*, 63, 624-628.

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 Journal*, 17, 609-614.

Riballo, E., Critchlow, S.E., Teo, S.H., Doherty, A.J., Priestley, A., Broughton, B., Kysela, B., Beamish, H., Plowman, N., Arlett, C.F., Lehmann, A.R., Jackson, S.P. and Jeggo, P.A. (1999) Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. *Curr Biol*, 19, 699-702.

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.

Riballo, E., Kuhne, M., Rief, N., Doherty, A., Smith, G.C., Recio, M.J., Reis, C., Dahm, K., Fricke, A., Krempler, A., Parker, A.R., Jackson, S.P., Gennery, A., Jeggo, P.A. and Lobrich, M. (2004) A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Mol Cell*, 16, 715-724.

Stiff, T., Shtivelman, E., Jeggo, P. and Kysela, B. (2004) AHNAK interacts with the DNA ligase IV-XRCC4 complex and stimulates DNA ligase IV mediated double stranded ligation. *DNA Repair*, 3, 245-256.

Valencia, M., Bentele, M., Vaze, M.B., Herrmann, G., Kraus, E., Lee, S.E., Schar, P. and Haber, J. (2001) NEJ1 controls non-homologous end joining in *Saccharomyces cerevisiae*. *Nature*, 414, 666-669.

van der Burg, M., van Veelen, L.R., Verkaik, N.S., Wiegant, W.W., Hartwig, N.G., Barendregt, B.H., Brugmans, L., Raams, A., Jaspers, N.G., Zdzienicka, M.Z., van Dongen, J.J. and van Gent, D.C. (2006) A new type of radiosensitive T-B-NK+

severe combined immunodeficiency caused by a LIG4 mutation. *J Clin Invest*, 116, 137-145.

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.

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.

Weterings, E. and van Gent, D.C. (2004) The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair (Amst)*, 3, 1425-1435.

FIGURE LEGENDS.

Figure 1. XLF is dispensable for DNA ligase IV stability and is a weaker binding partner of the stable LX complex.

(**A**) 1BR3 and 2BN primary human fibroblasts were analysed by immunofluorescence using antibodies against DNA Ligase IV, XRCC4 and XLF. Cells were counterstained with DAPI. (**B**) Left panel: 100 μg of whole cell extract (WCE) from control (48BRhT) and XLF-deficient (2BNhT) hTERT, immortalised fibroblasts were analysed by immunoblotting with α-DNA Ligase IV, α-XRCC4, and α-XLF antibodies. Immunoblots were reprobed with α-Ku70 antibody as a loading control. Panels A and B show that 2BN cells express normal levels of DNA Ligase IV and XRCC4 but no detectable XLF. Middle panel: 15 μg 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 μg of WCE from Chinese hamster ovary control (AA8) and XRCC4 deficient (XR1) cells were analysed by immunoblotting. XR-1 cells show reduced levels of DNA Ligase IV. XRCC4 and XLF expression was not examined since the antibodies used do not cross react with the rodent protein. Previous studies have shown XR-1 cells have no detectable XRCC4 (**C**) MRC5BIVA cells were transiently transfected with N-terminal FLAG-tagged XLF (F-XLF) or empty vector (F). 500 μg of WCE were incubated with α-FLAG antibodies and the immunoprecipitates 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 verify the level of XLF immunoprecipitated (bottom panel). The interaction between XLF and LX is reduced at 0.5M NaCl and abolished at 1M 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 proteins were 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 at 1 M NaCl.

Figure 2. XLF stimulates Ligase IV re-adenylation following ligation.

(**A**) LX complexes were treated with pyrophosphate (PPi) for 15 min at room temperature (RT). Following dialysis to remove PPi, 1 pmol LX was incubated with α-^{32}P-ATP in the presence or absence of 5 pmols XLF. XLF stimulated the LX adenylation activity 2.5 fold. Results represent the average and SE of two experiments. (**B**) LX was treated with PPi to de-adenylate LigIV. Following dialysis, 1 pmol LX was incubated with α-^{32}P-ATP, with or without 30 pmols XLF and 0.2 pmols DNA ends (445 bp ds pBluescript fragment). The level of LX adenylation was decreased in the presence of DNA, suggesting that ligation may cause LX de-adenylation. (**C**) 1 pmol LX was incubated with 0.2 pmols DNA ends (445 bp fragment) for 15 min to allow ligation and hence de-adenylation. α-^{32}P-ATP was then added in the presence or absence of 5 pmols XLF and samples were examined for adenylate complex formation. XLF enhanced the rate of adenylate complex formation ~3 fold. Whether XLF was present or absent during the pre-incubation step did not change the level of adenylate complex formation. Results are the average and SE of two experiments. Similar findings have been observed in additional experiments using a range of conditions. **D**) Samples were treated as in C and were examined for ligation by gel electrophoresis. Ligation occurred during the pre-incubation step and peaked by 15 mins. The gel shown is representative of three independent experiments (**E**) 0.2 pmols LX were incubated with 0.8 pmols DNA ends in the presence or absence of 1 pmol XLF without ATP for 15 mins and samples were examined for ligation. The presence of XLF did not impact upon the level of ligation. (**F**) 2 mg WCE from human pre-B control cells (Nalm6) were immunoprecipitated with α-XRCC4 antibodies and washed in buffer containing 1 or 0.12 M NaCl. Samples were treated with or without PPi in the presence or absence of ~35 pmols DNA ends prior to examination for adenylate complex formation. LX-αAMP is the adenylated LX complex. Immunoprecipitated samples were subjected to Western blot analysis using α-DNA Ligase IV antibodies to assess protein levels. The graph shows arbitrary units of intensity of adenylation, normalised to protein levels. The results shown are representative of two experiments.

Figure 3. XLF stimulates LX double stranded ligation in an ATP dependent and independent manner.

(**A**) 125 fmols LX complex were incubated with 60 fmols 5'-^{32}P-end-labelled DNA fragment (445bp) and increasing amounts of XLF. The ligation products were analysed by gel electrophoresis. The far-left panel shows substrate ligated by T4-ligase and non-ligated substrate as controls. XLF stimulated LX ligation activity (**B**) Increasing amounts of LX complexes were incubated with or without 17 pmols of GST-XLF in the absence (left panel) or presence (right panel) of ATP and 60 fmols 5'-^{32}P-end-labelled substrate. No multimers are formed in the absence of XLF using this amount of LX. XLF stimulated LX ligation in the absence of ATP (left panel) and further stimulation was observed in the presence of ATP (right panel). (**C**) 25 fmols 5'-^{32}P-end-labelled 445 bp substrate, 100 fmols biotinylated 445 bp substrate, 0.65 pmols DNA-PK and/or 17 pmols GST-XLF were incubated at RT for 15 min as indicated. The biotinylated substrate was pulled down using streptavidin beads and the presence of co-associated radioactivity was quantitated. 17 pmols GST-XLF shows the same level of bridging as endogenous 0.65 pmols DNA-PK. The results represent the mean and SD of 3 experiments (**D**) 5 fmols LX complexes were incubated with or without 15 pmols XLF and 60 fmols 5'-^{32}P-end-labelled 445 bp DNA fragment as indicated. Ligation was estimated at time points shown. Little ligation occurred in the absence of XLF (diamonds). XLF stimulated ligation in the absence of ATP but an increase in the maximum level of ligation was achieved in the presence of XLF and ATP. All ligation assays are representative of a single experiment but similar results have been observed in at least two additional experiments.

Figure 4. 2BN cells show a 3-fold reduced rate of DSB rejoining. (**A**) C-HA-XLF^{WT} (XLF), C-HA-XLF^{11insT} (11insT) and empty vector (HA) were transfected into MRC5BIVA cells. XLF was immunoprecipitated from 500 μg whole cell extract using α-HA antibodies. 50 μg WCE and a-HA immunoprecipitates (HA-IP) were analysed with α-HA or α-XLF antibodies. A smaller product could not be detected in cells transfected with either XLF or 11insT-XLF. (**B**) Human primary fibroblasts from control (1BR3), XLF-deficient (2BN) and LIG4 syndrome patients (180BR and 495BR) were analysed by immunofluorescence for the rate of loss of γ-H2AX foci after exposure to 3 Gy IR using α-γ-H2AX antibody. Results are the mean and SD of three experiments. The rate of DSB

repair in 2BN cells is similar to that of the LIG4 syndrome cell lines. (**C**) The rate of DSB repair following 3 Gy IR was also analyzed in mouse embryonic fibroblasts (MEFs) defective in DNA Ligase IV ($LigIV^{-/-}p53^{-/-}$) as in B. Compared with control MEFs, the $LigIV^{-/-}p53^{-/-}$ MEFs show no detectable rejoining up to 72 hours post irradiation (3 Gy). (**D**) Quantification of DSB rejoining over the first 6 hours following 3 Gy IR. The rate of repair in 1BR3 cells was approximately 5.7 DSBs per hour while 2BN cells only repaired 2.1 DSBs per hour.

FIGURE 1.

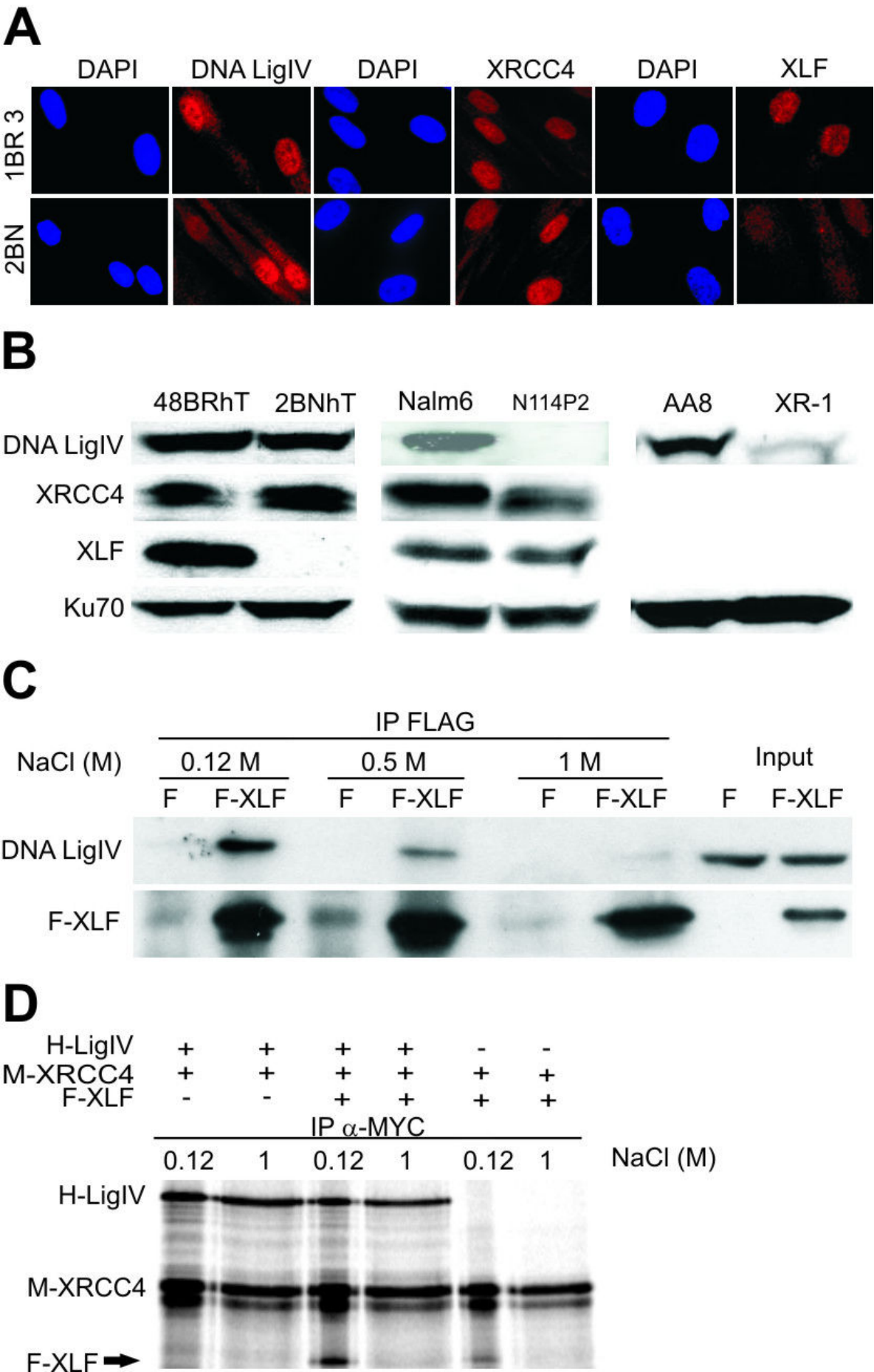

A
DAPI
DNA LigIV
DAPI
XRCC4
DAPI
XLF
1BR 3
2BN
B
48BRhT
2BNhT
Nalm6
N114P2
AA8
XR-1
DNA LigIV
XRCC4
XLF
Ku70
C
IP FLAG
NaCl (M)
0.12 M
0.5 M
1 M
Input
F
F-XLF
F
F-XLF
F
F-XLF
F
F-XLF
DNA LigIV
F-XLF
D
H-LigIV
M-XRCC4
F-XLF
IP α-MYC
0.12
1
0.12
1
0.12
1
NaCl (M)
H-LigIV
M-XRCC4
F-XLF

FIGURE 2.

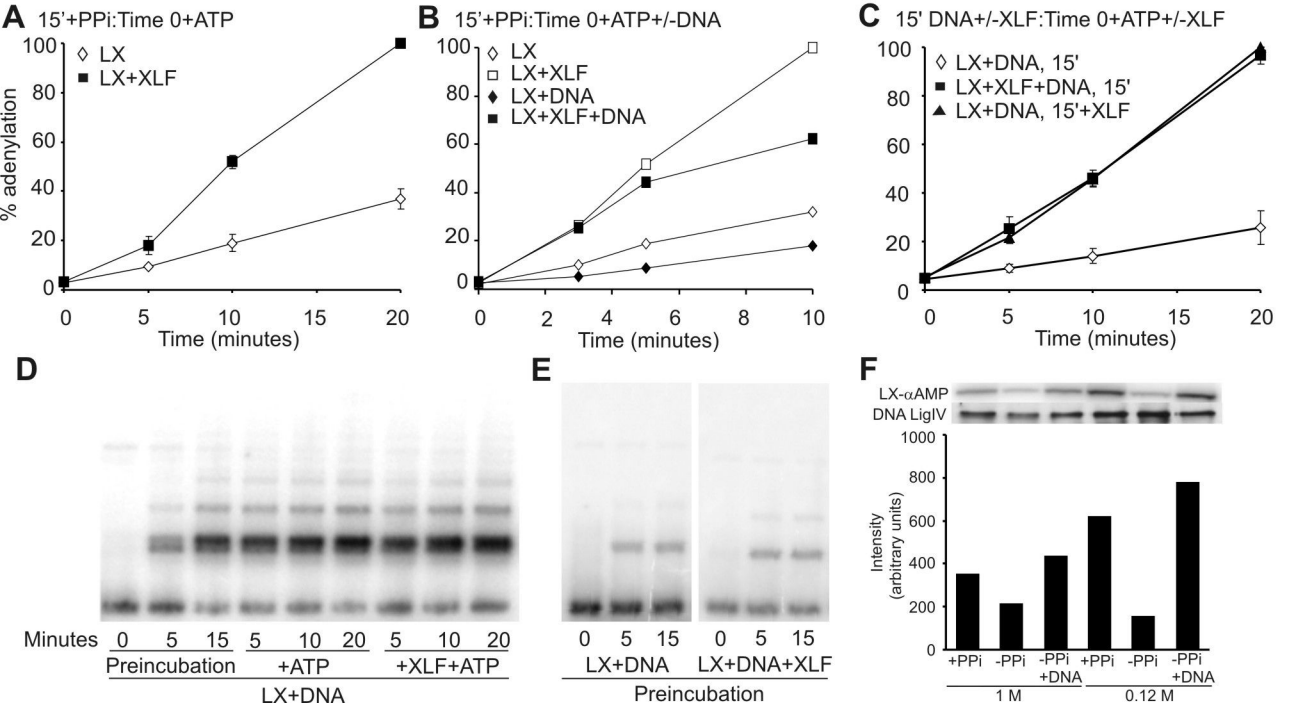

A
15'+PPi:Time 0+ATP
LX
LX+XLF
% adenylation
Time (minutes)
B
15'+PPi:Time 0+ATP+/-DNA
LX
LX+XLF
LX+DNA
LX+XLF+DNA
Time (minutes)
C
15' DNA+/-XLF:Time 0+ATP+/-XLF
LX+DNA, 15'
LX+XLF+DNA, 15'
LX+DNA, 15'+XLF
Time (minutes)
D
Minutes 0 5 15 5 10 20 5 10 20
Preincubation +ATP +XLF+ATP
LX+DNA
E
0 5 15 0 5 15
LX+DNA LX+DNA+XLF
Preincubation
F
LX-αAMP
DNA LigIV
Intensity (arbitrary units)
+PPi -PPi -PPi +DNA +PPi -PPi -PPi +DNA
1 M 0.12 M

FIGURE 3.

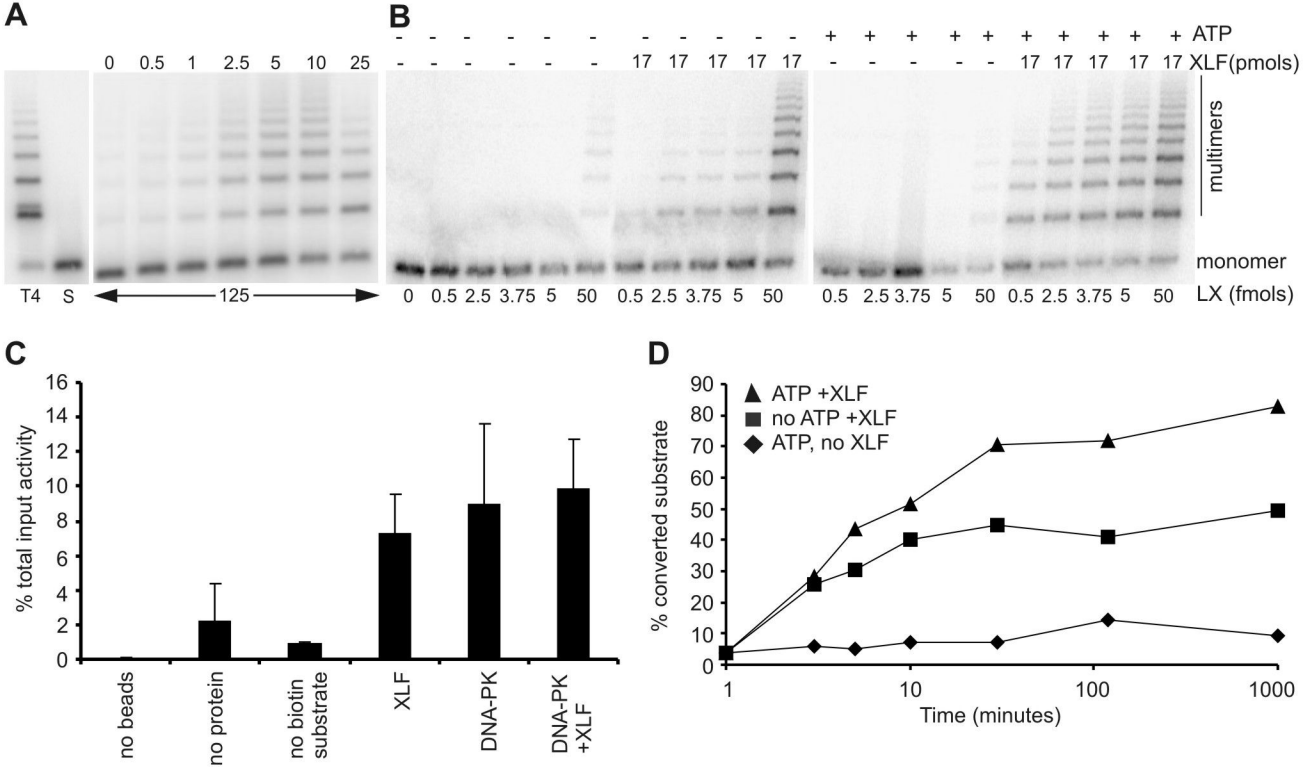
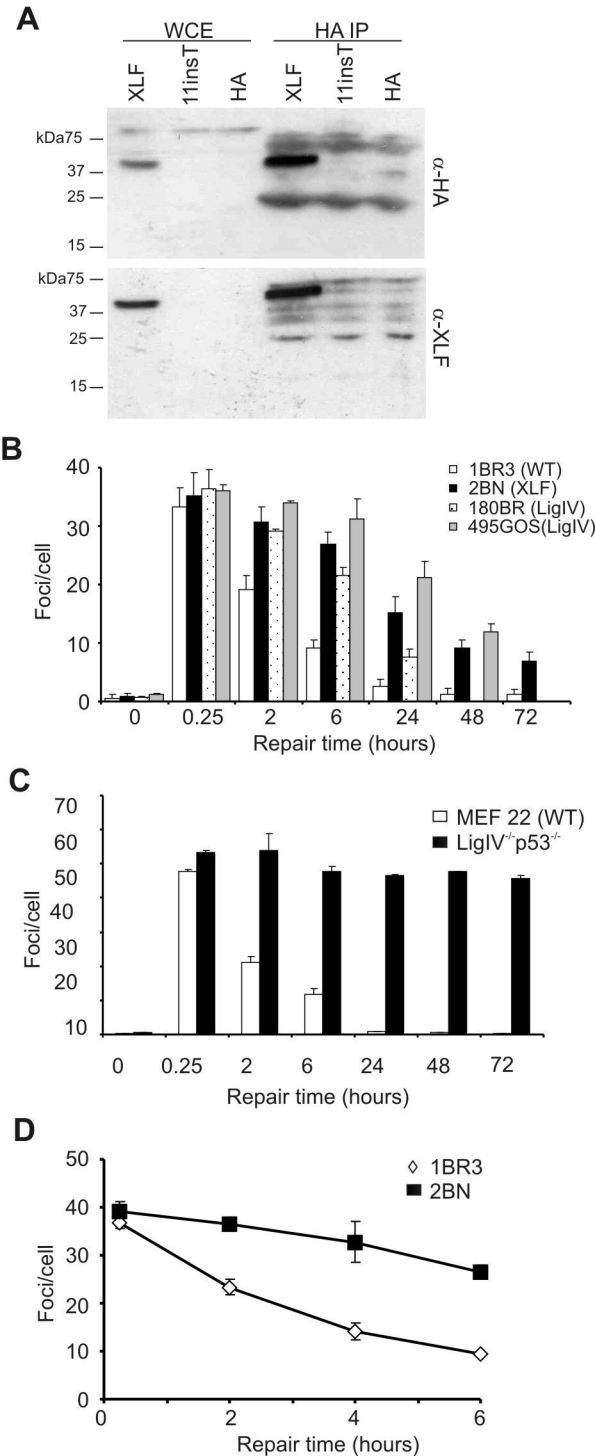

A
B
0 0.5 1 2.5 5 10 25
T4 S
125
- - - - - - - - - - - - + + + + + + + + + + ATP
- - - - - - 17 17 17 17 17 - - - - - 17 17 17 17 17 XLF(pmols)
multimers
monomer
0 0.5 2.5 3.75 5 50 0.5 2.5 3.75 5 50
0.5 2.5 3.75 5 50 0.5 2.5 3.75 5 50
LX (fmols)
C
% total input activity
16
14
12
10
8
6
4
2
0
no beads
no protein
no biotin substrate
XLF
DNA-PK
DNA-PK +XLF
D
% converted substrate
90
80
70
60
50
40
30
20
10
0
ATP +XLF
no ATP +XLF
ATP, no XLF
1
10
100
1000
Time (minutes)

FIGURE 4.


A
WCE
HA IP
XLF
11insT
HA
XLF
11insT
HA
kDa75
37
25
15
α-HA
kDa75
37
25
15
α-XLF
B
Foci/cell
1BR3 (WT)
2BN (XLF)
180BR (LigIV)
495GOS(LigIV)
0
0.25
2
6
24
48
72
Repair time (hours)
C
Foci/cell
MEF 22 (WT)
LigIV-/-p53-/-
0
0.25
2
6
24
48
72
Repair time (hours)
D
Foci/cell
1BR3
2BN
0
2
4
6
Repair time (hours)