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Monoubiquitination of the nonhomologous end joining protein XRCC4

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Abstract

Nonhomologous end joining is one of the major pathways by which cells repair double-strand breaks, and the XRCC4–DNA ligase IV complex is required for the ligation step. To better understand the regulation and stability of XRCC4 and DNA ligase IV, we investigated the ubiquitination status of these two proteins. We identified a predominantly monoubiquitinated form of XRCC4, and higher molecular weight forms of ubiquitinated XRCC4 were detected in lower abundance. In response to etoposide-induced DNA damage, ubiquitinated XRCC4 became more pronounced and was additionally phosphorylated. We confirmed that DNA ligase IV is unstable in the absence of XRCC4, with a half-life of approximately 30–90 min. Unlike XRCC4, we did not detect ubiquitinated forms of DNA ligase IV, and we found that the presence of XRCC4 stabilized DNA ligase IV more significantly than proteasome inhibitors. Monoubiquitination of XRCC4 may play a critical role in the regulation of nonhomologous end joining.

Keywords: XRCC4; Ubiquitin; Nonhomologous end joining; DNA ligase IV; DNA repair

The nonhomologous end joining (NHEJ) DNA repair pathway is a major pathway of double-strand break repair in mammalian cells. Proteins involved in the NHEJ pathway include DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ku70, Ku80, XRCC4, and DNA ligase IV (Lig4) [1]. Many of the NHEJ proteins are also essential for the lymphocyte process of V(D)J recombination. XRCC4 and Lig4 are critical components of both pathways and, as such, are often the focus of investigation to better understand their regulation and interactions.

XRCC4 is a nuclear phosphoprotein that has a core functional domain consisting of amino acids 18–204 [2,3]. XRCC4 is constitutively phosphorylated, and in response to irradiation, is further phosphorylated in a DNA-PK-dependent process [4,5]. Lig4 protein forms a tight complex with XRCC4 [6], with stoichiometric analyses suggesting a 2:1 XRCC4–Lig4 complex [7,8]. XRCC4 has been shown to stimulate Lig4 activity [9] and is required to stabilize Lig4 [10]. XRCC4 and Lig4 are essential for genomic stability, with a deficiency in either XRCC4 or Lig4 resulting in premature senescence, ionizing radiation sensitivity, and defective V(D)J recombination. Mice deficient in either protein also exhibit embryonic lethality and defective neurogenesis [11,12]. In addition, clinical reports of Lig4 syndrome have described a rare autosomal recessive condition caused by mutations in the Lig4 gene, characterized by microcephaly, growth retardation, developmental delay, and immunodeficiency [13].

Lig4 has been shown to be unstable in the absence of XRCC4, with XRCC4 stabilizing Lig4 at the protein level, rather than affecting gene expression [10]. Lig4 stability is also affected by DNA-PK-dependent phosphorylation [14]. The mechanism by which XRCC4 facilitates Lig4 stability has not been determined. Controlled protein degradation of one or both proteins would be an efficient mechanism through which NHEJ and V(D)J recombination could be tightly regulated.

Proteasome-dependent degradation is a major mechanism for targeted protein degradation and involves a cascade of enzymatic reactions, leading to the covalent attachment of ubiquitin via internal lysine residues or at the N-terminus of target proteins [15]. Polyubiquitin chains, classically linked via lysine 48 of ubiquitin, are

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recognized by proteasomes located throughout the cell. The ubiquitinated protein is then hydrolyzed and free ubiquitin regenerated. As one of the major pathways of protein degradation, we have investigated the involvement of ubiquitin-dependent degradation in Lig4 regulation.

Modification of proteins by ubiquitin does not exclusively lead to targeted protein degradation, because ubiquitination can play a regulatory role. Instead of modification by polyubiquitin chains, proteins can be monoubiquitinated at single or multiple sites. Also, the polyubiquitin chains can be linked via lysines in ubiquitin other than lysine 48. Multiple regulatory roles for ubiquitin modifications have been demonstrated, including nuclear trafficking, kinase activation, endocytosis, and histone regulation [16].

The involvement of ubiquitin in DNA repair processes is well documented. For example, Fanconi Anemia D2 protein (FANCD2) monoubiquitination is associated with its localization to nuclear foci following DNA damage, along with other DNA repair factors [17]. In this study, we examined the ubiquitination status of XRCC4 and Lig4 to determine whether ubiquitin is involved in the regulation of the XRCC4–Lig4 complex.

Experimental procedures

Plasmids. C-terminal FLAG-tagged mouse XRCC4 (pCMV-Tag4-mXRCC4) was derived from *Eco*RI/*Xho*I digestion of pcDNAI.MXR [11] and ligation into pCMV-Tag4a (Stratagene). N-terminal FLAG-tagged mouse Lig4 (pYW315) was derived from mouse Lig4 in pcDNA3 [12], followed by digestion with *Nhe*I and *Xho*I to recover the C-terminal domain. The N-terminal region was PCR amplified before ligation into pCMV-Tag2A (Stratagene). C-terminal histidine (His)-tagged human XRCC4 (pYW101) was derived by *Xba*I/*Xho*I digestion of pBMM42 (pET28a-hXRCC4) [18] and ligation into pcDNA3.1/Hygro (−) (Invitrogen). The C-terminal HA-tagged human XRCC4 construct (pKF134) was derived from pYW101 by insertion of a *Hin*dIII/*Xho*I PCR fragment and a double-stranded oligonucleotide containing the HA tag. The N-terminal FLAG-tagged human Lig4 construct (pCN220) was derived from pDR119 [18] by blunting an *Xho*I/*Nco*I insert and ligating into pCMV-Tag2A (Stratagene), followed by insertion of a PCR fragment containing a FLAG tag. All constructs were sequenced. These plasmids were kindly provided: pBMM42 [18] from Martin Gellert, pDR119 [18] from Mauro Modesti, the hemagglutinin (HA)-tagged ubiquitin expression construct, pMT123 (HA-Ub) [19], from Dirk Bohmann, the FLAG-tagged SGK construct (SGK-1-FLAG) [20] from Suzanne Conzen, and the FLAG-tagged ubiquitin construct (pCMV10-3xFLAG-ubiquitin) [21] from Jeffrey Benovic.

Cell culture. XR-1 cells (defective in XRCC4) were cultured in F12K media (Cellgro) with 10% fetal calf serum (FCS), 1% glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, 20 μM HEPES, 0.1 mM nonessential amino acids (Invitrogen), and 50 μM β-mercaptoethanol. MCF7 cells were cultured in minimum essential media Eagle's media (Invitrogen) with 10% FCS, glutamine, and penicillin/streptomycin. Human embryonic kidney (HEK) 293 T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FCS, glutamine, and penicillin/streptomycin.

Cells were treated with ammonium chloride (2.5 mM), chloroquine (100 μM), lactacystin (10 μM), MG132 (Boronic) (1–10 μM), *N*-acetyl-leu-leu-norleu-al (ALLN) (50 μM), or *N*-acetyl-leu-leu-met-al (ALLM) (50 μM) (Sigma) for 8 h. Cells were treated with etoposide (75–100 μM) (MP Biomedicals) as indicated.

In vivo ubiquitination assays. Constructs were transfected using Lipofectin (Invitrogen) or Fugene6 (Roche). Protease inhibitors were added 8 h prior to harvesting. Etoposide was added for the indicated time periods prior to harvesting. After a 48 h incubation, cells were harvested on ice and washed in cold phosphate-buffered saline (PBS). Cells were lysed in Tris lysis buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 2% Triton X-100 (Fisher), and 10 mM EDTA) containing 1% NP-40 and protease inhibitors. After centrifugation, lysates were precleared with isotype specific immunoglobulins and protein A–agarose (Invitrogen). Immunoprecipitation was carried out with equal amounts of protein as determined by the Bradford assay. FLAG-tagged proteins were immunoprecipitated with either mouse IgG (Sigma) or mouse anti-FLAG M2-conjugated agarose (Sigma). HA-human XRCC4 was immunoprecipitated with rabbit polyclonal anti-XRCC4 antibody (Serotec) and subsequent incubation with protein A–agarose. Following extensive washing with Tris lysis buffer containing 0.1% SDS and protease inhibitors and a final wash with cold PBS, immunoprecipitates were eluted by boiling 5 min in loading buffer. Phosphatase treatments were carried out following immunoprecipitation and washing. Beads were incubated with 400 U (Fig. 2) or 800 U (Fig. 3) Lambda Protein Phosphatase (λ-PPase) (New England Biolabs) in 1 × λ-PPase buffer (50 mM Tris–HCl, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, and 0.01% Brij 35, pH 7.5) with 2 mM $MnCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM PMSF, and 1 mM benzamidine for 1 h at 30 °C. Beads were then washed with cold PBS and eluted as described above.

MCF7 cells were lysed in NET lysis buffer (50 mM Tris–HCl (Fisher) pH 7.5, 120 mM NaCl (Fisher), 0.5% v/v NP-40 (USB), 5 mM EDTA (Fisher)), containing 1 μg/ml aprotinin (Sigma), 1 mM phenylmethylsulfanyl fluoride (PMSF) (MP Biomedicals), 1 μg/ml leupeptin (Sigma), 1 mM benzamidine (Sigma), 25 mM sodium fluoride (Sigma), 50 μM ALLN, and ALLM.

Antibodies and immunological methods. Antibodies used for immunoblotting: anti-HA-horseradish peroxidase (HRP) (Roche; Clone 3F10) 1:500 or 1:1000, anti-FLAG-HRP (Sigma) 1:500 or 1:1000, monoclonal anti-p53 (Calbiochem; Ab6 DO1) 1:300, monoclonal anti-poly-ADP ribose polymerase (PARP-1) (Serotec) 1:500, monoclonal anti-β-actin (Sigma; Clone AC-15) 1:2000, polyclonal anti-XRCC4 (Serotec; AHP387) 1:1000, monoclonal anti-Lamin B (Oncogene Research Products; Clone Ab-1) 1:500.

Cycloheximide mediated block of novel protein synthesis. XR-1 cells were transfected with FLAG-mouse Lig4 or cotransfected with FLAG-mouse XRCC4. HEK 293T cells were transfected with FLAG-tagged human Lig4 or cotransfected with His-tagged human XRCC4. Following a 48 h incubation period, cells were treated with 25–50 μg/ml cycloheximide (CHX), harvested at indicated timepoints, and protein levels were analyzed by immunoblotting or immunoprecipitation followed by immunoblotting. If protease inhibitors were used, cells were pretreated with inhibitor for 1 h prior to addition of CHX. Cells were then incubated in the presence of both CHX and the inhibitor, and harvested as described.

Pulse-chase labelling of cells. XR-1 cells were transfected with FLAG-mouse Lig4 or cotransfected FLAG-mouse XRCC4. Following a 48 h incubation period, cells were starved for 1 h in methionine/cysteine free media (Invitrogen) and pulse-labelled with 200 μCi/ml [^{35}S]methionine/cysteine mix (Redivue-promix, Amersham) for 2 h. Cells were washed and chased in complete media, supplemented with 2 mM methionine and cysteine. Cells were harvested at indicated timepoints, lysed, and immunoprecipitated with FLAG-conjugated agarose. Precipitates were resolved by SDS–PAGE and visualization of XRCC4 and Lig4 carried out by autoradiography.

Results

Exogenously expressed XRCC4 is monoubiquitinated

To analyze XRCC4 ubiquitination, a FLAG-tagged mouse XRCC4 construct and an HA-ubiquitin construct were expressed in XR-1 cells. XRCC4 was immunoprecipitated and analyzed for ubiquitin conjugation by immunoblotting with anti-HA antibody. As shown in Fig. 1A,

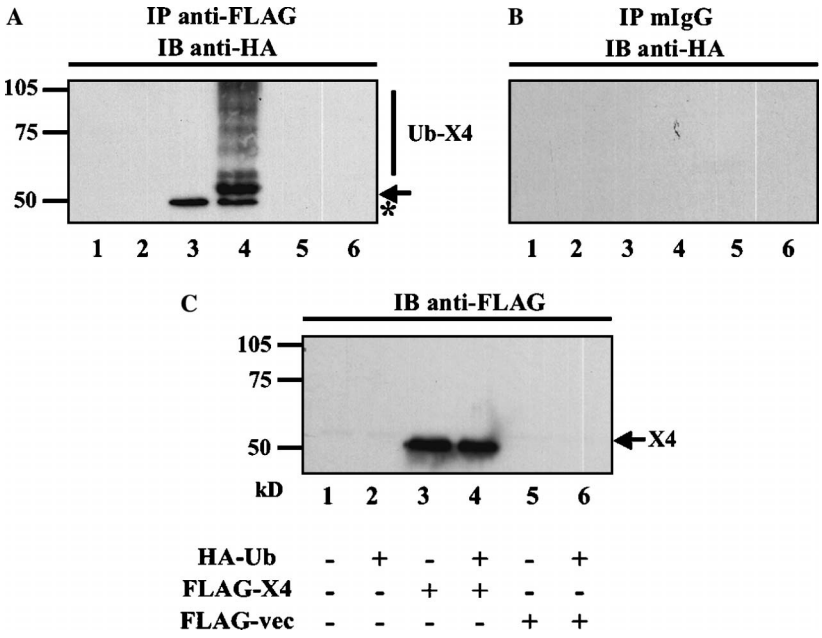

Fig. 1. Monoubiquitinated and higher-molecular weight ubiquitinated forms of XRCC4 are detected in cells expressing exogenous XRCC4. C-terminal FLAG-tagged mouse XRCC4 (FLAG-X4) or FLAG vector (FLAG-vec) were transfected into XR-1 cells, or cotransfected with an HA-tagged ubiquitin expression vector (HA-Ub). XRCC4 was immunoprecipitated with FLAG-conjugated agarose (A) and mouse IgG (mIgG) was used as an isotype control (B). After SDS–PAGE and Western blotting, ubiquitinated proteins were visualized with anti-HA antibody (IB anti-HA) (lane 4, A). The 50 kDa band in lanes 3 and 4 (A) is due to cross-reaction of the HA antibody with immunoprecipitated protein (marked by an asterisk). However, the higher-molecular weight forms generate a specific signal, only detected when XRCC4 is immunoprecipitated from cells transfected with HA-Ub. The monoubiquitinated form of XRCC4 is the most prominent band and is marked by an arrow (A). Successful XRCC4 immunoprecipitation was confirmed by Western blot analysis of immunoprecipitates using anti-FLAG antibody (IB anti-FLAG) (C). IB, immunoblot; IP, immunoprecipitation; X4, XRCC4. Results have been confirmed in more than six independent experiments.

mouse XRCC4 was specifically ubiquitinated. Specific immunoprecipitation was confirmed by the presence of isotype controls (Fig. 1B). The prominent modified form of XRCC4 is consistent in size with monoubiquitinated XRCC4. In addition, several higher molecular weight forms were detected. Similar experiments in HEK 293T, HeLa, and MCF7 cells using His-tagged human XRCC4 also identified ubiquitinated XRCC4 (data not shown). While ubiquitinated forms of XRCC4 were identified with anti-HA antibody, direct visualization of XRCC4 with anti-FLAG antisera detected only unmodified XRCC4 (Fig. 1C). The HA detection is more sensitive than the FLAG detection by more than 100-fold (data not shown), so XRCC4 was also tagged with HA, as discussed later, to demonstrate that the ubiquitinated protein is XRCC4. The identification of a monoubiquitinated form of XRCC4 suggests that ubiquitin may be involved in the regulation of XRCC4.

DNA damage induces XRCC4 ubiquitination

We investigated XRCC4 ubiquitination following DNA damage, because XRCC4 is a component of the NHEJ DNA repair complex. HEK 293T cells transfected with His-tagged human XRCC4 and HA-tagged ubiquitin were treated with etoposide for 0, 8, 16, and 24 h prior to harvesting. Samples were immunoprecipitated with anti-XRCC4 sera, then analyzed by immunoblotting with anti-HA sera to detect ubiquitinated XRCC4, and with anti-His sera to confirm XRCC4 immunoprecipitation (Fig. 2A). As etoposide treatment lengthens, ubiquitinated XRCC4 was detected with increasing intensity (Fig. 2A, upper left panel). An additional form of ubiquitinated XRCC4 was also detected (Fig. 2A, upper left panel, lane 3 compared to lane 6). This correlates with a shift in XRCC4 (Fig. 2A, lower left panel), which is likely mediated by DNA damage-induced phosphorylation of XRCC4 [4]. Although the ubiquitinated form of the protein was not visible in the exposure shown in the lower panel after anti-His antibody detection in this specific time-course experiment, we have observed the ubiquitinated form of XRCC4 using anti-His detection of the His-tagged XRCC4 protein and anti-HA detection of HA-tagged XRCC4 in multiple experiments (data not shown and Fig. 3).

Immunoprecipitation input (IP input) from each sample was analyzed by Western blotting with anti-HA, anti-PARP-1, anti-XRCC4, and anti-β-actin antibodies (Fig. 2B). HA detection confirmed equal ubiquitin expression in all samples, with β-actin used as a further loading control. PARP-1 cleavage, mediated by caspase-3, is an early marker of apoptosis. Anti-XRCC4 antibody was used to determine equal expression of XRCC4 in transfected cells and to monitor XRCC4 cleavage [4]. Etoposide treatment for 24 h resulted in minimal PARP-1 and XRCC4 cleavage (Fig. 2B), indicating that the majority of cells are not undergoing apoptosis but are still in a repair phase.

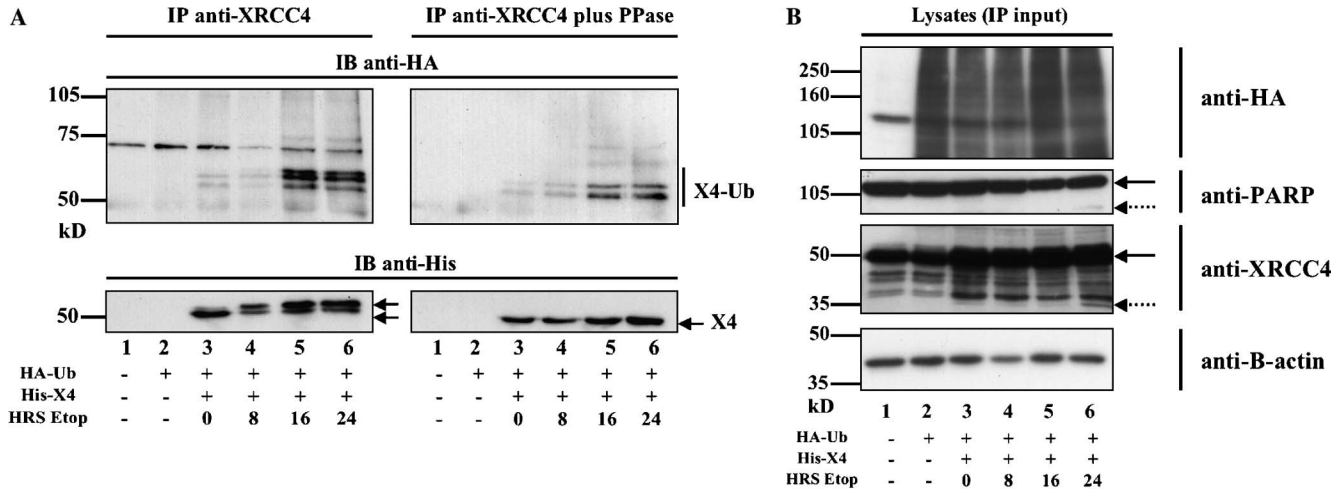

Fig. 2. DNA damage enhances XRCC4 ubiquitination, in combination with phosphorylation of XRCC4. HEK 293T cells transfected with HA-tagged ubiquitin (HA-Ub) and human His-XRCC4 (His-X4) expression constructs were treated with etoposide for the indicated number of hours (HRS Etop). (A) Samples were immunoprecipitated with anti-XRCC4 antisera and then treated with phosphatase (plus PPase). Control samples were mock treated. Following SDS–PAGE, ubiquitinated proteins were detected by immunoblotting with anti-HA antibody, and XRCC4 immunoprecipitation was confirmed by immunoblotting with anti-His antisera. Arrows indicate constitutively expressed XRCC4, and higher molecular weight XRCC4 that has been modified following DNA damage. Etoposide-mediated induction of XRCC4 ubiquitination has been confirmed in more than 10 independent experiments and phosphatase experiments were performed more than four times. (B) Controls for DNA damage response: Lysates (IP input) were analyzed by Western blotting for HA (Ub), PARP-1, XRCC4, and β-actin expression. Full-length PARP-1 and XRCC4 are indicated by the solid arrows, while cleaved proteins are indicated by broken arrows.

These results clearly indicate that XRCC4 ubiquitination is enhanced following etoposide treatment.

Phosphorylated XRCC4 induced by DNA damage is ubiquitinated

To determine if ubiquitinated XRCC4 is also phosphorylated, we carried out DNA damaging treatments as described above, but with an additional step of phosphatase treatment following immunoprecipitation (Fig. 2A, right-hand panels). We confirmed that etoposide treatment leads to the phosphorylation of XRCC4. With increasing duration of drug treatment, there was a clear shift in XRCC4, with two forms detected (Fig. 2A, lower left panel). After phosphatase treatment, however, XRCC4 resolved as a single band on SDS–PAGE, confirming that this was a phosphorylation modification (lower right panel). XRCC4 is constitutively phosphorylated [5,22] and as a result, this dephosphorylated XRCC4 migrates slightly below XRCC4 that has not undergone phosphatase treatment. Ubiquitinated XRCC4 becomes more prominent following DNA damage, with an additional species identified (Fig. 2A, upper left panel). Phosphatase treatment confirmed that this additional form was both ubiquitinated and phosphorylated, since after phosphatase treatment it was not detected (Fig. 2A, upper right panel). Monoubiquitinated XRCC4 and a second ubiquitinated form of XRCC4 were still detected following phosphatase treatment. We have shown that XRCC4 is both phosphorylated and ubiquitinated following etoposide-induced DNA damage. Further studies will be needed to determine if ubiquitination is dependent on the phosphorylation state of XRCC4.

Endogenous XRCC4 is ubiquitinated

To demonstrate that the ubiquitinated protein that we observed is indeed XRCC4 and not another protein of similar size that coimmunoprecipitated with XRCC4, we generated an HA-tagged XRCC4 to be used along with a FLAG-tagged ubiquitin construct. We transfected HEK293T cells with expression constructs for HA-tagged XRCC4, FLAG-tagged ubiquitin, or both. The cells were then treated with etoposide for 24 h or mock treated, followed by immunoprecipitation. The results demonstrate multiple critical points. First, the effect of etoposide treatment was again observed as shown in Fig. 3B, lane 4 compared to lane 2, or Figs. 3B and C, lane 8 compared to lane 6. The second point from this experiment is that the ubiquitinated XRCC4 was clearly seen with HA detection as shown in Fig. 3B, lanes 2–5, and lanes 8 and 9. Because XRCC4 is known to be a highly phosphorylated protein that is further phosphorylated in response to DNA damage, it was critical to demonstrate that the bands that we attributed to ubiquitinated forms of XRCC4 were not simply phosphorylated forms of the protein. As shown in Fig. 3B, lanes 3, 5, and 9, the band was still present after phosphatase treatment. The ubiquitinated form of XRCC4 was apparent in sample number 7 even without immunoprecipitation (Fig. 3D, lane 5). We were also able to detect the ubiquitinated form of HA-tagged XRCC4 in cells that contained only endogenous ubiquitin (Fig. 3B, lanes 2–5).

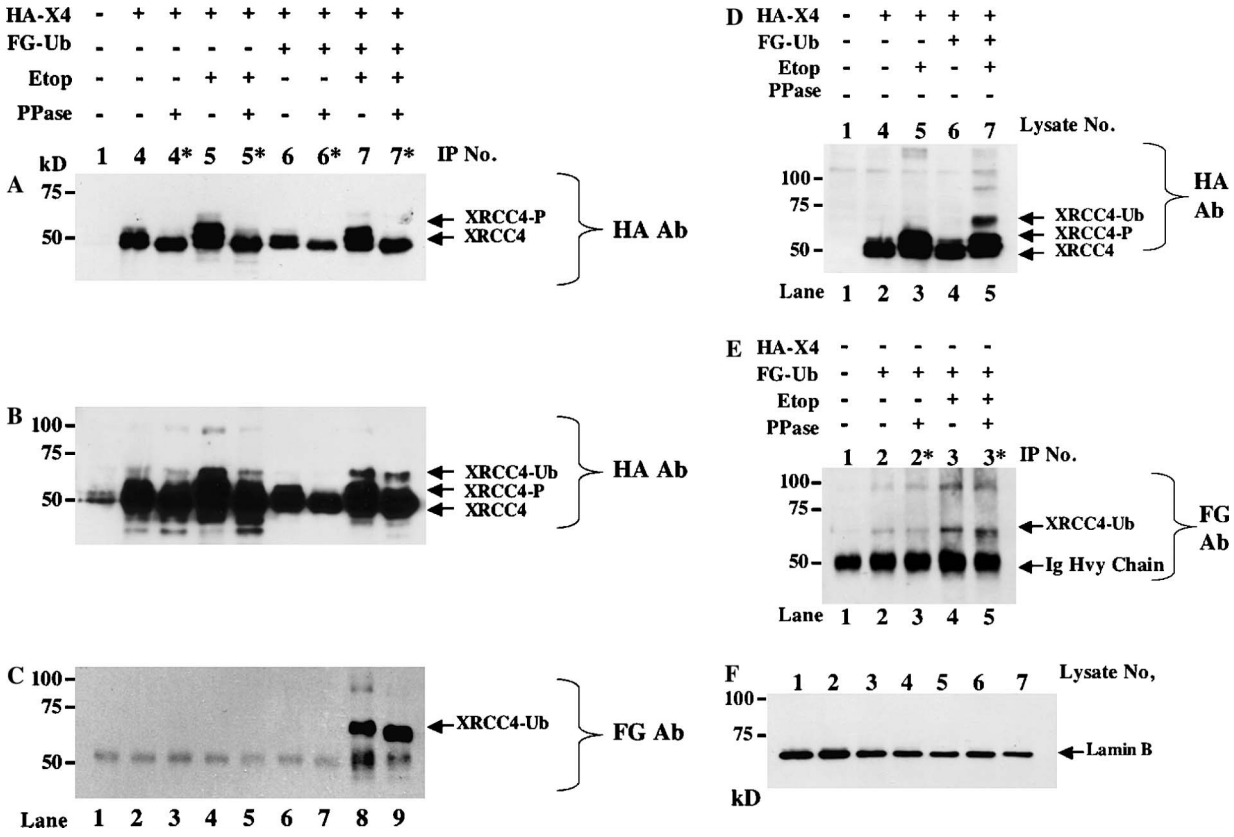

Fig. 3. Endogenous XRCC4 is monoubiquitinated. HEK 293T cells were transfected with expression plasmids for human HA-tagged XRCC4, FLAG-tagged ubiquitin, or both. After a 24 h incubation, cells were treated with etoposide (Etop) for 24 h or mock treated. All samples were immunoprecipitated with anti-XRCC4 antisera and then treated with phosphatase (sample numbers marked with an asterisk) or mock treated. After SDS–PAGE analysis, Western blots were analyzed with anti-HA (A,B) or anti-FLAG (C) antisera. Samples are numbered 1–7 consistently for (A–F). (A) A short exposure is shown to demonstrate the effect of phosphatase treatment on phosphorylated XRCC4. (B) A longer exposure of the same blot is shown to demonstrate the detection of monoubiquitinated XRCC4 after immunoblotting for HA, a method that detects the XRCC4 protein. (C) Detection of monoubiquitinated XRCC4 after immunoblotting for FLAG, a method that detects ubiquitin. The ubiquitinated XRCC4 protein band is slightly reduced in size by PPase treatment, but the bands have a higher molecular weight than those of dephosphorylated and nonubiquitinated XRCC4. The effect of etoposide treatment is most clearly seen in lanes 8 and 9 of C. Samples 4 and 5 do not contain exogenous ubiquitin, so the HA-tagged XRCC4 is ubiquitinated with endogenous ubiquitin as demonstrated in lanes 2–5 of (B). (D) The ubiquitinated XRCC4 in sample 7, which was treated with etoposide is abundant enough to see in the cell lysate, and did not require immunoprecipitation as shown in lane 5. (E) Endogenous XRCC4 was demonstrated to be ubiquitinated in samples 2 and 3 in lanes 2–5. The effect of etoposide treatment on endogenous XRCC4 ubiquitination is evident in lanes 4–5 compared to lanes 2–3. Immunoprecipitation experiments using rabbit IgG confirmed the specificity of the signal. (F) Equal volume of precleared cell lysates was examined by immunoblotting for Lamin B as a loading control. Ig Hvy chain; Immunoglobulin heavy chain. Results were confirmed in more than three independent experiments.

While we have clearly seen ubiquitinated forms of XRCC4 with both mouse and human proteins, using FLAG-tagged, HA-tagged, and His-tagged XRCC4 in combination with either HA-tagged or FLAG-tagged ubiquitin, the XRCC4 protein was overexpressed in those experiments. To determine if endogenous XRCC4 is ubiquitinated, we transfected cells with only the ubiquitin construct and used anti-XRCC4 antisera to immunoprecipitate endogenous protein. The third point of this experiment is that endogenous XRCC4 was indeed ubiquitinated as shown in Fig. 3E in untreated cells (lanes 2 and 3). The ubiquitination was enhanced in etoposide-treated cells (Fig. 3E, lanes 4 and 5). Immunoprecipitation experiments using rabbit IgG as a control were performed to confirm the specificity of the endogenous XRCC4-ubiquitin signal (data not shown).

In summary, we detect a monoubiquitinated form of XRCC4 and clearly show that the result we observed was not due to a protein that coimmunoprecipitated with XRCC4. In addition, the ubiquitinated form of XRCC4 was observed in cells containing endogenous levels of both XRCC4 and Lig4, and etoposide treatment increased the ubiquitination of the endogenous XRCC4 protein.

XRCC4 is critical for Lig4 stability

We next investigated whether Lig4 instability, in the absence of XRCC4, was due to ubiquitin targeting Lig4 for degradation via the proteasome pathway. FLAG-mouse Lig4 was coexpressed with HA-Ub, in the presence or absence of XRCC4, in XR-1 cells. We did not see a clear signal for Lig4 ubiquitination, either in the presence or

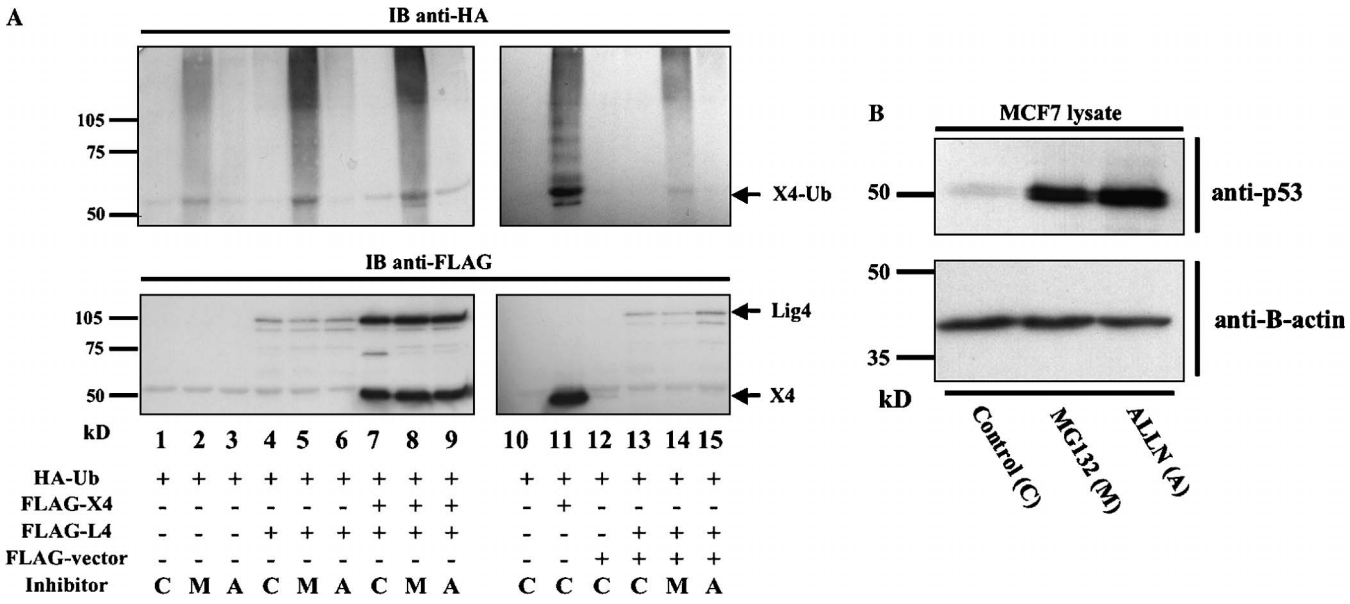

Fig. 4. The presence of XRCC4 is critical for Lig4 stability. (A) XR-1 cells transfected with indicated combinations of HA-Ub, FLAG-tagged mouse XRCC4 (FLAG-X4), FLAG-tagged mouse Lig4 (FLAG-L4), and FLAG-vector were treated with vehicle control (C), MG132 (M) or ALLN (A) for 8 h. FLAG-conjugated agarose was used to immunoprecipitate Lig4 and XRCC4. Following SDS–PAGE, blots were visualized using anti-HA antibody (upper panels). Successful immunoprecipitation of XRCC4 and Lig4 was confirmed using anti-FLAG antibody (lower panel). Results were confirmed in more than three independent experiments. (B) Control for effective proteasome inhibition. Lysates from MCF7 cells treated in parallel with MG132 and ALLN were analyzed for p53 and β-actin expression.

absence of XRCC4. XRCC4 and Lig4 expression was confirmed by immunoblotting anti-FLAG (Fig. 4, lower panels). The absence of XRCC4 in XR-1 cells resulted in decreased Lig4 protein, but this was not accompanied by detection of ubiquitinated Lig4. XR-1 cells transfected with HA-Ub alone (Fig. 4A, lanes 1 and 10), and FLAG-vector plus HA-Ub (Fig. 4A, lane 12) were both negative for ubiquitin detection after immunoprecipitation, as expected. FLAG-XRCC4 plus HA-Ub was also transfected (Fig. 4A, lane 11), providing a positive control in this experiment, as shown by the detection of ubiquitinated XRCC4 (Fig. 4A, upper panel, lane 11). In addition, human Lig4 has been expressed in HEK 293T cells and, again, no clear Lig4 ubiquitination signal was detected (data not shown).

It is possible that ubiquitinated Lig4 is present, but below the level of detection of this assay. However, inhibition of the proteasome would be expected to result in the accumulation of ubiquitinated species. We have therefore investigated Lig4 ubiquitination following treatment with two different proteasome inhibitors MG132 and ALLN. XR-1 cells transfected as described above were treated with MG132 or ALLN for 8 h prior to harvesting. After immunoprecipitation, samples were analyzed by immunoblotting with anti-HA and anti-FLAG sera (Fig. 4A). There was a significant increase in non-specific ubiquitin detection after treatment with MG132 (Fig. 4A, upper panel, lane 2). The increase in ubiquitin in samples where Lig4 was expressed (Fig. 4A, upper panel, lanes 5, 8, and 14) did not, therefore, appear to be above background levels. Furthermore, Lig4 ubiquitination was not detected after treatment with ALLN (lanes 6, 9, and 15). These data indicate that either Lig4 is not significantly ubiquitinated in the cell, or that the level of ubiquitinated Lig4 present, even in the presence of proteasome inhibitors, is below the level of detection in this assay. Increased Lig4 expression was detected when XRCC4 was coexpressed. However, use of the inhibitors had no visible effect on the levels of Lig4 or XRCC4 expressed. As a control, p53 levels were monitored in extracts prepared from MCF7 cells, which had been treated in parallel with MG132 or ALLN. p53 has a half-life of approx 20–30 min [23]. Lysates were prepared and analyzed for p53 and β-actin expression (Fig. 4B), with p53 clearly stabilized by both inhibitors.

XRCC4 is a stable protein and Lig4 is unstable in the absence of XRCC4

While it has been reported that XRCC4 stabilizes Lig4, and is thought to stabilize it at the protein level [10], the kinetics of protein turnover have not been established, so we performed half-life assays. After transient transfection of constructs encoding XRCC4 and Lig4, cells were treated with CHX to prevent new protein synthesis, and harvested at time intervals up to 24 h following CHX addition. XRCC4 was stable over the 24 h period. Lig4, in the presence of XRCC4, was stable over these timepoints (Fig. 5A, lower panel), but in the absence of XRCC4, Lig4 had a half-life of approximately 30–90 min (Fig. 5A, upper panel). Classic pulse-chase analyses (Fig. 5B) were in agreement with CHX experiments, demonstrating a reduced half-life of Lig4 in the absence of XRCC4.

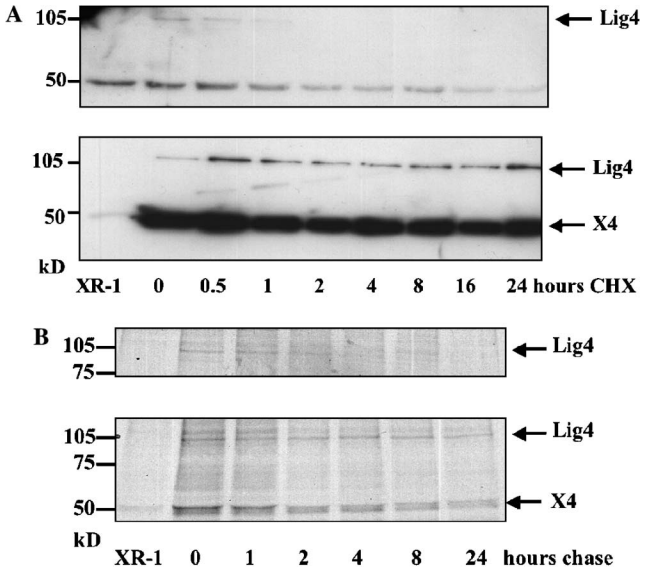

Fig. 5. Lig4 is unstable in the absence of XRCC4, with a reduced half-life. (A) Half-life of Lig4 and XRCC4 determined by cycloheximide (CHX) treatment. XR-1 cells were transfected with either FLAG-tagged mouse Lig4 (FLAG-L4) (upper panel) or cotransfected with FLAG-tagged Lig4 and FLAG-tagged mouse XRCC4 (FLAG-X4) (lower panel). After a 48 h incubation, cells were treated with CHX for the indicated times and lysates were immunoprecipitated with FLAG-conjugated agarose. Equal amounts of protein, as determined by Bradford assay, were used per timepoint. Precipitates were then analyzed by SDS–PAGE, and both XRCC4 and Lig4 were detected by immunoblotting using anti-FLAG antibodies. Untransfected cells (lane labelled XR-1) underwent the same procedure as a control for immunoprecipitation specificity. Three independent experiments were performed. (B) Half-life of Lig4 and XRCC4 determined by ^{35}S pulse-chase experiment. XR-1 cells were transiently transfected with either FLAG-tagged mouse Lig4 (FLAG-L4) (upper panel) or cotransfected with FLAG-tagged mouse Lig4 and FLAG-tagged mouse XRCC4 (FLAG-X4) (lower panel). Approximately 48 h following transfection, cells underwent pulse-chase labelling. Cells were harvested at the indicated timepoints and then immunoprecipitated with FLAG-conjugated agarose. Precipitates were resolved by SDS–PAGE, and XRCC4 and Lig4 were visualized by autoradiography. Untransfected XR-1 cells served as a control (lane labelled XR-1, upper and lower panels).

We also used MG132 in CHX half-life experiments. MG132 had a slight effect on the stability of Lig4 (Fig. 6), but the effect of XRCC4 on Lig4 stability was greater than the effect of MG132. HEK 293T cells were transfected with FLAG-tagged human Lig4, alone (Fig. 6A) or with His-tagged XRCC4 (Fig. 6B). After a 48 h incubation, cells were treated with CHX to follow protein turnover. In parallel, cells were pretreated with MG132 for 1 h before addition of CHX. Cells were harvested at the indicated timepoints and lysates examined for Lig4. The short-lived protein serum and glucocorticoid-induced protein kinase (SGK) [35], which is known to be stabilized by the MG132 inhibitor, was used as a control for this study to demonstrate the activity of the reagents (Fig. 6C) and for comparison with a protein that is clearly affected by proteasomal inhibition. This protein half-life experiment was also performed using XR-1 cells that do not express endogenous XRCC4, and identical results were obtained.

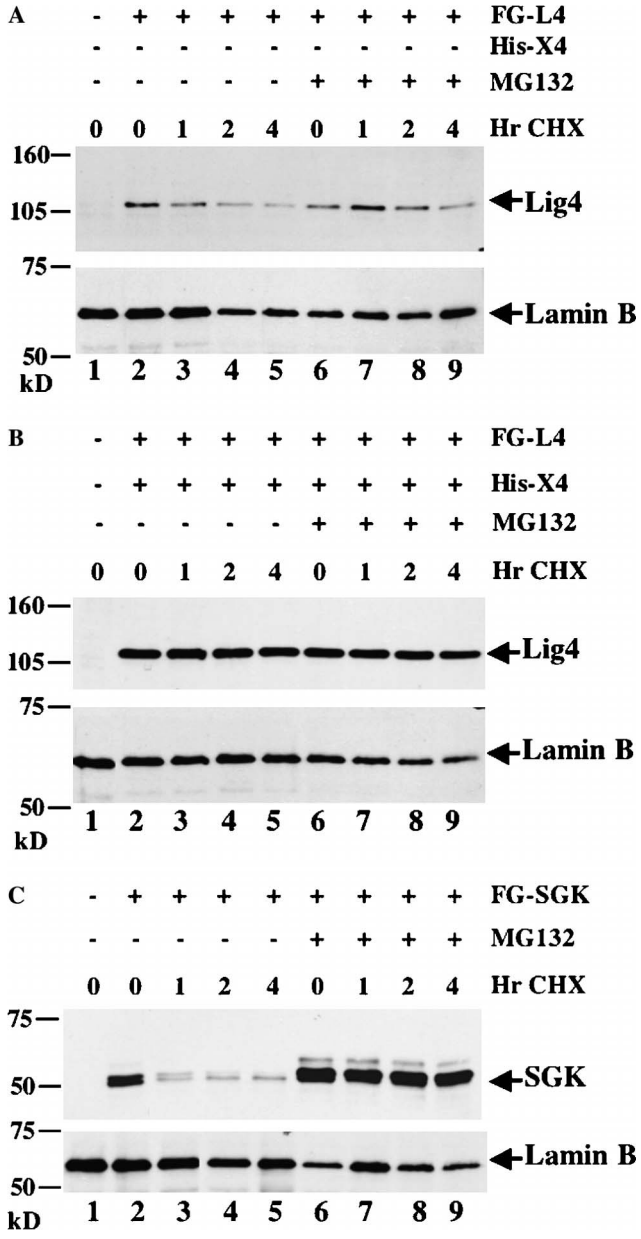

Fig. 6. Effect of XRCC4 on Lig4 half-life is greater than effect of proteasome inhibitor MG132. Only a slight increase in Lig4 stability was observed after inhibition of the proteasome with MG132 compared to the strong effect of the presence of XRCC4. HEK 293T cells were transfected with FLAG-tagged human Lig4, alone (A) or in combination with His-tagged human XRCC4 (B). After 48 h, cells were treated with cycloheximide (CHX). In parallel, cells were pretreated with the proteasome inhibitor MG132 for 1 h before addition of CHX. Cells were harvested at the indicated timepoints, then lysates were separated by SDS–PAGE gel and analyzed for Lig4. Lamin B served as a loading control. The top panel of (A) and the top panel of (B) were developed together on the same film for equal exposure times and comparison of levels. Likewise, the bottom panel of (A) and the bottom panel of (B) were developed together on the same film. The short-lived protein serum and glucocorticoid-induced protein kinase (SGK), which is known to be stabilized by the MG132 inhibitor was used as a control. HEK 293T cells were transfected with a FLAG-tagged SGK expression construct and the cells were then treated as described above. Equal volumes of cell lysates were run on two separate gels, followed by Western blot analysis for SGK or Lamin B. Results were confirmed in more than three independent experiments. These experiments were also performed in XR-1 cells, which lack functional XRCC4, and identical results were obtained.

XRCC4 stabilizes Lig4, but a series of protease inhibitors did not visibly stabilize Lig4 in the absence of XRCC4

We investigated the effect of other protease inhibitors on Lig4 stability. Cells expressing Lig4 in the presence or absence of XRCC4 were treated with a panel of protease inhibitors for 8 h. Inhibitors included the cathepsin inhibitors ammonium chloride and chloroquine, the specific proteasome inhibitor lactacystin, and the broad range inhibitors ALLN (effective against proteasome, calpain, and cathepsin degradation) and ALLM (effective against calpain and cathepsin degradation). When Lig4 was expressed in the absence of XRCC4 (Fig. 7, upper panel), treatment with inhibitors did not stabilize the protein. In the presence of XRCC4 (Fig. 7, middle panel), Lig4 protein levels were significantly increased. Treatment with proteasome-specific inhibitors also had no effect on XRCC4 protein expression, further suggesting that XRCC4 ubiquitination is not a degradation signal. Proteasome inhibitors were effective as demonstrated by the ability to stabilize p53 (Fig. 7, lower panel). p53 can also be degraded by mechanisms other than the proteasome, thus there is also increased p53 expression following treatment with alternative inhibitors.

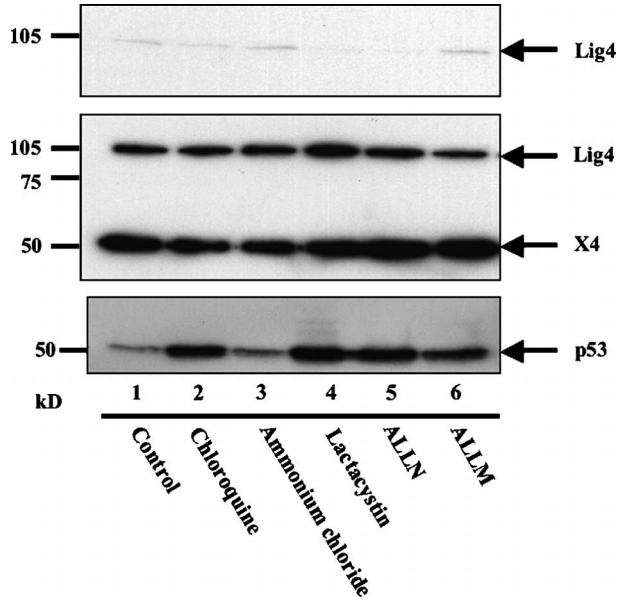

Fig. 7. The presence of XRCC4 has a greater effect on Lig4 stability than inhibition of calpain, cathepsin, and proteasome-dependent degradation pathways. XR-1 cells were transiently transfected with FLAG-mouse Lig4 (FLAG-L4) alone (upper panel) or cotransfected with FLAG-mouse Lig4 and FLAG-mouse XRCC4 (FLAG-X4) (middle panel). Approximately 48 h following transfection, cells were treated with proteasome inhibitors for 8 h. Samples were immunoprecipitated with FLAG-agarose and analyzed by SDS–PAGE. Immunoblotting with anti-FLAG antibody detected XRCC4 and Lig4. MCF7 cells were treated with inhibitors, lysates resolved by SDS–PAGE, and p53 detected by immunoblotting (lower panel). All inhibitors were used in at least three independent experiments, with similar results.

Discussion

Although ubiquitin is best known as a signal for protein degradation, modification of proteins by ubiquitin has significant regulatory functions as well [16]. Using multiple constructs in several cell lines, we identified a monoubiquitinated form of XRCC4. A relatively small proportion of endogenous XRCC4 was ubiquitinated; however, there was a significant increase in the amount of ubiquitinated XRCC4 following etoposide treatment. Because XRCC4 is a stable protein, and monoubiquitination was the predominant modification identified, it is likely that XRCC4 ubiquitination has a non-proteasomal role. Additionally, treatment with proteasome inhibitors did not lead to XRCC4 protein accumulation, further supporting a non-degradative signalling function for XRCC4 ubiquitination.

The interdependence of ubiquitination and phosphorylation has been reported in the literature [24,25]. XRCC4 is a known phosphoprotein, with additional phosphorylation occurring following DNA damage by irradiation [4]. It has not yet been examined if the ubiquitination of XRCC4 is dependent on a specific phosphorylation state of XRCC4 or if XRCC4 ubiquitination changes during the cell cycle. It has been reported that XRCC4 and Lig4 are not uniformly in a tight complex throughout the cell cycle [26], and we have observed cell-cycle-specific phosphorylation of XRCC4, independent of any DNA damage (Y. Wang and K. Frank, unpublished observations), making the possibility of a cell cycle effect on ubiquitination a reasonable consideration. Our results regarding the stability of Lig4 are consistent with those previously reported [10], as we were able to detect only low amounts of Lig4 in the absence of XRCC4, even when we transiently overexpressed the protein. We found no clear evidence of Lig4 ubiquitination in the presence or absence of XRCC4 nor after proteasome inhibition. It is possible that ubiquitinated Lig4 is eliminated rapidly, making it below the detection limit of our system. Proteasome-specific inhibitors were able to extend the Lig4 half-life slightly, but their use did not result in the level of stabilization seen with XRCC4 coexpression. Lig4 stability is affected by the phosphorylation state of the protein, and several additional forms were detected when the phosphorylation site was mutated [14], leading us to speculate that a specific protease may be responsible for targeted degradation of Lig4.

In addition to phosphorylation and ubiquitination, sumoylation affects multiple cellular processes including transcription, nuclear transport, signal transduction, and DNA repair [27]. Using the Abgent SUMOplot sumoylaton calculator (http://abgent.com/tool/sumoplot), several potential sumoylation sites were identified within XRCC4. We speculate that XRCC4 may be regulated through sumoylation in addition to monoubiquitination and phosphorylation.

In summary, while investigating the regulation of the XRCC4-Lig4 complex, we have identified a monoubiquitinated form of XRCC4, as well as additional ubiquitinated

forms of XRCC4. Ubiquitination of XRCC4 is enhanced following etoposide treatment, and DNA damage-induced phosphorylated XRCC4 is also ubiquitinated. How exactly phosphorylation and ubiquitination might connect to regulate XRCC4 function remains to be determined. Ubiquitination of XRCC4 could affect a number of functions of the protein, including the interaction with Lig4, the interaction with other components of the nonhomologous end joining pathway, the interaction of XRCC4 with DNA, or the intracellular localization of XRCC4, specifically the transportation of XRCC4 to DNA damage foci. In addition, our investigations have demonstrated that XRCC4 stabilizes Lig4 protein, extending its half-life, with an effect that is more significant than that seen with proteasome inhibitors. Future studies will include an examination of the XRCC4 ubiquitination in response to other DNA damaging agents and an investigation of the possible sumoylation of XRCC4.

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References

[1] M.L. Hefferin, A.E. Tomkinson, Mechanism of DNA double-strand break repair by non-homologous end joining, DNA Repair (Amst) 4 (2005) 639–648.
[2] R. Mizuta, H.L. Cheng, Y. Gao, F.W. Alt, Molecular genetic characterization of XRCC4 function, Int. Immunol. 9 (1997) 1607–1613.
[3] U. Grawunder, D. Zimmer, P. Kulesza, M.R. Lieber, Requirement for an interaction of XRCC4 with DNA ligase IV for wild-type V(D)J recombination and DNA double-strand break repair in vivo, J. Biol. Chem. 273 (1998) 24708–24714.
[4] Y. Matsumoto, N. Suzuki, N. Namba, N. Umeda, X.J. Ma, A. Morita, M. Tomita, A. Enomoto, S. Serizawa, K. Hirano, K. Sakaia, H. Yasuda, Y. Hosoi, Cleavage and phosphorylation of XRCC4 protein induced by X-irradiation, FEBS Lett. 478 (2000) 67–71.
[5] Y. Yu, W. Wang, Q. Ding, R. Ye, D. Chen, D. Merkle, D. Schriemer, K. Meek, S.P. Lees-Miller, DNA-PK phosphorylation sites in XRCC4 are not required for survival after radiation or for V(D)J recombination, DNA Repair (Amst) 2 (2003) 1239–1252.
[6] K.J. Lee, J. Huang, Y. Takeda, W.S. Dynan, DNA ligase IV and XRCC4 form a stable mixed tetramer that functions synergistically with other repair factors in a cell-free end-joining system, J. Biol. Chem. 275 (2000) 34787–34796.
[7] B.L. Sibanda, S.E. Critchlow, J. Begun, X.Y. Pei, S.P. Jackson, T.L. Blundell, L. Pellegrini, Crystal structure of an Xrcc4-DNA ligase IV complex, Nat. Struct. Biol. 8 (2001) 1015–1019.
[8] M. Modesti, M.S. Junop, R. Ghirlando, M. van de Rakt, M. Gellert, W. Yang, R. Kanaar, Tetramerization and DNA ligase IV interaction of the DNA double-strand break repair protein XRCC4 are mutually exclusive, J. Mol. Biol. 334 (2003) 215–228.
[9] U. Grawunder, M. Wilm, X. Wu, P. Kulesza, T.E. Wilson, M. Mann, M.R. Lieber, Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells, Nature 388 (1997) 492–495.
[10] M. Bryans, M.C. Valenzano, T.D. Stamato, Absence of DNA ligase IV protein in XR-1 cells: evidence for stabilization by XRCC4, Mutat. Res. 433 (1999) 53–58.
[11] Y. Gao, Y. Sun, K.M. Frank, P. Dikkes, Y. Fujiwara, K.J. Seidl, J.M. Sekiguchi, G.A. Rathbun, W. Swat, J. Wang, R.T. Bronson, B.A. Malynn, M. Bryans, C. Zhu, J. Chaudhuri, L. Davidson, R. Ferrini, T. Stamato, S.H. Orkin, M.E. Greenberg, F.W. Alt, A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis, Cell 95 (1998) 891–902.
[12] K.M. Frank, J.M. Sekiguchi, K.J. Seidl, W. Swat, G.A. Rathbun, H.L. Cheng, L. Davidson, L. Kangaloo, F.W. Alt, Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV, Nature 396 (1998) 173–177.
[13] T.I. Ben-Omran, K. Cerosaletti, P. Concannon, S. Weitzman, M.M. Nezarati, A patient with mutations in DNA Ligase IV: clinical features and overlap with Nijmegen breakage syndrome, Am. J. Med. Genet. A 137 (2005) 283–287.
[14] Y.G. Wang, C. Nnakwe, W.S. Lane, M. Modesti, K.M. Frank, Phosphorylation and regulation of dna ligase iv stability by DNA-dependent protein kinase, J. Biol. Chem. 279 (2004) 37282–37290.
[15] C.M. Pickart, D. Fushman, Polyubiquitin chains: polymeric protein signals, Curr. Opin. Chem. Biol. 8 (2004) 610–616.
[16] R.L. Welchman, C. Gordon, R.J. Mayer, Ubiquitin and ubiquitin-like proteins as multifunctional signals, Nat. Rev. Mol. Cell. Biol. 6 (2005) 599–609.
[17] M. Grompe, A. D'Andrea, Fanconi anemia and DNA repair, Hum. Mol. Genet. 10 (2001) 2253–2259.
[18] M. Modesti, J.E. Hesse, M. Gellert, DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity, EMBO J. 18 (1999) 2008–2018.
[19] M. Treier, L.M. Staszewski, D. Bohmann, Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain, Cell 78 (1994) 787–798.
[20] D.R. Brickley, C.A. Mikosz, C.R. Hagan, S.D. Conzen, Ubiquitin modification of serum and glucocorticoid-induced protein kinase-1 (SGK-1), J. Biol. Chem. 277 (2002) 43064–43070.
[21] A. Marchese, J.L. Benovic, Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting, J. Biol. Chem. 276 (2001) 45509–45512.
[22] S.E. Critchlow, R.P. Bowater, S.P. Jackson, Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV, Curr. Biol. 7 (1997) 588–598.
[23] M.S. Rodriguez, J.M. Desterro, S. Lain, D.P. Lane, R.T. Hay, Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation, Mol. Cell Biol. 20 (2000) 8458–8467.
[24] M.V. Chernov, L.J. Bean, N. Lerner, G.R. Stark, Regulation of ubiquitination and degradation of p53 in unstressed cells through C-terminal phosphorylation, J. Biol. Chem. 276 (2001) 31819–31824.
[25] D. Kornitzer, A. Ciechanover, Modes of regulation of ubiquitin-mediated protein degradation, J. Cell Physiol. 182 (2000) 1–11.
[26] M.R. Przewloka, P.E. Pardington, S.M. Yannone, D.J. Chen, R.B. Cary, In vitro in vivo interactions of DNA ligase IV with a subunit of the condensin complex, Mol. Biol. Cell 14 (2003) 685–697.
[27] R.T. Hay, SUMO: a history of modification, Mol. Cell 18 (2005) 1–12.