

# DNA Ligase IV Mutations Identified in Patients Exhibiting Developmental Delay and Immunodeficiency

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## Summary

**DNA ligase IV functions in DNA nonhomologous end-joining and V(D)J recombination. Four patients with**

features including immunodeficiency and developmental and growth delay were found to have mutations in the gene encoding DNA ligase IV (*LIG4*). Their clinical phenotype closely resembles the DNA damage response disorder, Nijmegen breakage syndrome (NBS). Some of the mutations identified in the patients directly disrupt the ligase domain while others impair the interaction between DNA ligase IV and Xrcc-4. Cell lines from the patients show pronounced radiosensitivity. Unlike NBS cell lines, they show normal cell cycle checkpoint responses but impaired DNA double-strand break rejoining. An unexpected V(D)J recombination phenotype is observed involving a small decrease in rejoining frequency coupled with elevated imprecision at signal junctions.

## Introduction

DNA double-strand breaks (DSBs) are a deleterious form of DNA damage that can result in loss or rearrangement of genomic material, events leading to cell death or carcinogenesis. DSBs are induced by ionizing radiation (IR), but they also arise as intermediates in normal endogenous processes including DNA replication, and meiotic and V(D)J recombination. The induction of DSBs elicits a cascade of events including break sensing, signal transduction, and effector functions. The cellular mechanisms involved include DNA repair, cell cycle checkpoint arrest, and apoptosis, which together serve to limit the generation of mutations and the proliferation of damaged cells.

The hereditary disorders ataxia telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) result from defects in mechanisms responding specifically to DSBs (Carney et al., 1998; Savitsky et al., 1995; Varon et al., 1998). A-T and NBS patients display clinical radiosensitivity, immunodeficiency, and elevated cancer incidence (Taylor et al., 1996). A-T is a neurodegenerative disorder characterized by progressive cerebellar ataxia and telangiectasia. NBS patients develop neither ataxia nor telangiectasias and are instead characterized by microcephaly, growth retardation, and mild to moderate mental retardation (Weemaes et al., 1993; Wegner et al., 1999).

ATM (ataxia telangiectasia mutated protein), the protein defective in A-T, is a serine-threonine kinase that is central to the signal transduction process induced by DSBs (Lavin and Khanna, 1999; Savitsky et al., 1995). The ATM kinase is activated following exposure to IR and phosphorylates proteins involved in cell cycle checkpoint control and DNA repair. Cell lines derived from A-T patients show marked radiosensitivity and an inability to effect cell cycle checkpoints following radiation exposure (Lavin and Khanna, 1999).

NBS cell lines fail to produce the protein nibrin, which complexes with two additional proteins, hMre11 and hRad50, and is responsible for their nuclear localization (Carney et al., 1998; Varon et al., 1998). These complexes accumulate in nuclear foci near the site of DSBs (Desai-

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Mehta et al., 2001; Nelms et al., 1998). Like A-T, NBS cell lines are radiosensitive and demonstrate impaired cell cycle checkpoint arrest (Lim et al., 2000; Stewart et al., 1999; Yamazaki et al., 1998). Interestingly, hMre11 is defective in families with ataxia telangiectasia-like disorder (ATLD), which is characterized by mild A-T clinical features (Stewart et al., 1999). Taken together, the data suggest that ATM and the Nibrin/hMre11/hRad50 complex play an early role in a signal transduction pathway responding to DSBs that activates both repair and checkpoint functions.

The major mechanism for DSB repair in mammalian cells is DNA nonhomologous end-joining (NHEJ) (Jeggo, 1998). Five proteins function in NHEJ in mammalian cells. Three are components of the DNA-dependent protein kinase (DNA-PK) complex, the two subunits of Ku (Ku70 and Ku80) and a catalytic subunit termed DNA-PKcs. The remaining two components are Xrcc4 and DNA ligase IV. In the current model of NHEJ, Ku binds to DNA double-strand ends and recruits DNA-PKcs, activating its catalytic activity. The Xrcc4/DNA ligase IV complex carries out the final ligation step. Cell lines defective in NHEJ are radiosensitive with impaired ability to rejoin DSBs as monitored by pulse field gel electrophoresis (PFGE) (Jeggo, 1998). NHEJ is also required for V(D)J recombination, a site-specific rearrangement occurring in developing T and B cells (Taccioli et al., 1993). Although A-T and NBS cell lines are highly radiosensitive, they are proficient in both DSB rejoining and V(D)J recombination and therefore do not appear to be defective in NHEJ (Foray et al., 1997; Girard et al., 2000; Hsieh et al., 1993; Yeo et al., 2000). Conversely, rodent cell lines lacking NHEJ components do not display cell cycle checkpoint defects (Jeggo, 1998). Together, these findings demonstrate a separation between these damage response mechanisms.

Only one patient with a defect in NHEJ has been described to date (Riballo et al., 1999). This patient was homozygous for a missense mutation in the gene encoding DNA ligase IV that impairs its adenylation and ligation activities. The patient did not display any overt immunodeficiency or other clinical characteristics but developed leukemia at age 14 and dramatically overresponded to radiotherapy. The lack of reported clinical features was surprising since DNA ligase IV knockout mice are embryonic lethal but was ascribed to residual function of the mutant protein (Riballo et al., 2001). Although important in identifying DNA ligase IV as contributing to human radiosensitivity, this finding provided no evidence for a role of NHEJ proteins in developmental processes.

In this study, we describe two independent but complementary approaches that have led to the identification of additional patients with defects in DNA ligase IV. One strategy, based on the prediction that patients defective in NHEJ would show impaired V(D)J recombination, involved an examination of radiosensitivity in cell lines derived from patients with uncharacterized immunodeficiency. The second approach was to screen for mutations in candidate genes among patients who were diagnosed as having NBS based on their clinical presentations but who did not harbor mutations in *NBS1*. The patients described in this study are quite distinct from the previously characterized DNA ligase IV defec-

tive patient and define a syndrome, designated LIG4 syndrome, associated with chromosomal instability, immunodeficiency, and developmental delay.

## Results

### 411BR, a Cell Line Derived from an Immunodeficiency Patient, Is Radiosensitive and Defective in DSB Rejoining

To identify patients with defects in NHEJ, we examined cell lines from patients with uncharacterized immunodeficiency for radiosensitivity. 411BR, a primary skin fibroblast line derived from one such patient, displayed similar radiosensitivity to an A-T (AT5BI) and an NBS (CZD82CH) cell line (Figure 1A). The 9 year-old patient (411BR) showed pancytopenia and additional clinical features that overlap with those displayed by NBS (Table 1). However, Western blot analysis revealed normal levels of nibrin and RT-PCR sequencing of *NBS1* and *MRE11* cDNA failed to reveal any mutations, ruling out a diagnosis of NBS or ATLD (data not shown). We next examined DSB rejoining in 411BR using PFGE. A pronounced decrease was observed both in the initial rate of rejoining and in the final extent of DSBs rejoined after 24 hr (Figure 1B). This decrease was similar to that seen in NHEJ defective cell lines, including the previously characterized ligase IV defective line, 180BR, and distinct from the much smaller defect found in A-T and NBS cell lines (Foray et al., 1997; Girard et al., 2000).

### 411BR Has Mutations in the DNA Ligase IV Gene

The DSB rejoining defect suggested that 411BR might be defective in a component of the NHEJ machinery. The function of proteins involved in this process was examined using 411BRneo, an SV40-transformed derivative of 411BR. All three components of the DNA-PK complex (Ku70, Ku80, and DNA-PKcs) were expressed normally. Normal levels of ds DNA end-binding activity with the mobility expected of Ku were observed and normal DNA-PK activity was measured by phosphorylation of a p53-derived peptide (data not shown). To monitor DNA ligase IV activities, we exploited previous findings that DNA ligase IV is immunoprecipitated in active form by anti-Xrcc4 (Critchlow et al., 1997; Grawunder et al., 1997). Similar ligase IV levels were observed by Western blotting of anti-Xrcc4 immunoprecipitates derived from control (1BR3neo) and 411BRneo cell extracts (Figure 1C). However, an adenylation assay performed on the immunoprecipitates revealed no detectable ligase IV-adenylate complex formation in 411BR cell extracts (Figure 1D). A nick ligation assay also failed to show any activity above the background levels observed using a ligase IV null preB cell line (N114-P2) (Figure 1E). These data indicate that, although DNA ligase IV is efficiently expressed in 411BR cells and can interact with Xrcc4, its activities are impaired, suggesting that 411BR carries a mutation in *LIG4* or *XRCC4*. The cDNAs of both genes were sequenced by RT-PCR; no mutations were identified in *XRCC4* but three homozygous changes were found in *LIG4*, 8C>T, 26C>T, and 833G>A. The 833G>A mutation results in an R278H substitution, identical to that previously identified in 180BR (Riballo et al., 1999). The R278 residue lies within

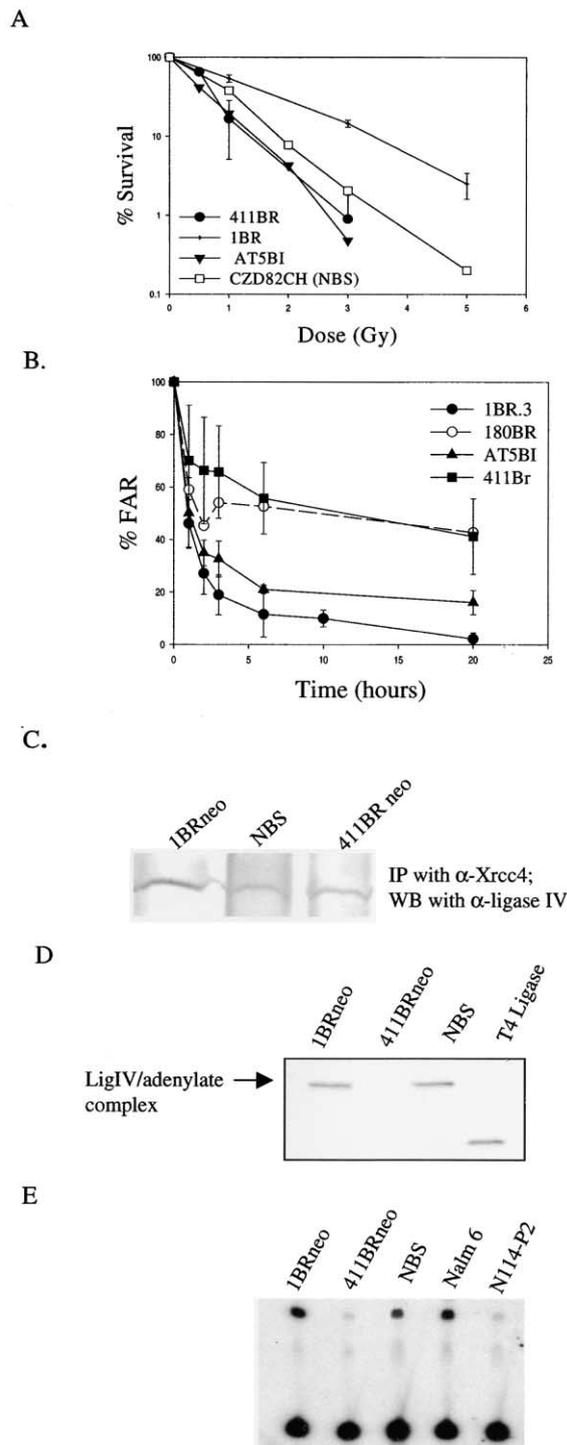


Figure 1. Cellular Phenotype of 411BR Cells

(A) 411BR cells are radiosensitive. Survival was assessed by colony formation following  $\gamma$ -ray exposure. 1BR3 is a control fibroblast cell line; AT5BI and CZD82CH are A-T and NBS lines, respectively. The results for all lines represent the mean and standard deviation (where shown) of at least three experiments.

(B) 411BR cells are defective in DSB rejoining. Following exposure to 40 Gy  $\gamma$ -rays, DSB rejoining was monitored by PFGE. The fraction of activity released (FAR) represents the radioactivity that enters the gel, divided by the total activity (well plus lane). The results are presented as the ratio of the FAR remaining at the specified time compared to the FAR at time 0. The FAR for unirradiated cells is

a highly conserved motif encompassing the active site, and the substitution significantly impairs ligase IV function (Riballo et al., 2001). The other two mutations are predicted to result in N-terminal amino acid substitutions, A3V and T9I. All three nucleotide substitutions were confirmed in genomic DNA from the patient. A parent and one unaffected sibling were heterozygous for the three mutations.

#### Mutations in *LIG4* Confer an NBS-like Phenotype

We have previously described patients with clinical characteristics of NBS who do not have mutations in *NBS1* and produce normal levels of nibrin protein (Cero-saletti et al., 1998; Hiel et al., 2001). From 37 such patients screened, three additional patients with mutations in *LIG4* were identified, two affected siblings from one family (patients 2303 and 2304) and a third unrelated patient (99P0149). Like patient 411BR, the patients displayed unusual facial features, microcephaly, growth and/or developmental delay, pancytopenia, and various skin abnormalities (Table 1).

Both affected siblings, patients 2303 and 2304, are compound heterozygotes for two truncating mutations, 1738C>T (R580X) and 2440C>T (R814X). The former is predicted to result in loss of the C terminus encompassing the two Brct domains present in *LIG4*, while the latter lies in the region between the two Brct domains. The third patient, 99P0149, is a compound heterozygote for the mutation 1406G>A resulting in an amino acid substitution, G469E, and the R814X mutation. We also observed seven individuals heterozygous for the 26C>T substitution, two of whom were also heterozygous for 8C>T. No patients homozygous for these substitutions, like 411BR, were observed. There were also two patients heterozygous for the silent substitution 1704T>C and another heterozygous for 2465C>T (5822L).

SSCP analysis of *XRCC4* from these "NBS-like" patient samples failed to detect any mutations that might have a functional impact. A previously reported polymorphism, 400T/C (I/T134), was detected in one patient (Ford et al., 2000).

#### Impaired Expression of DNA Ligase IV in *LIG4* Syndrome Patients

To examine the cellular impact of the truncating mutations, we used a lymphoblastoid cell line (LCL), LB 2304, derived from patient 2304, and a primary skin fibroblast cell line, FB2303, derived from the sibling. FB2303 showed similar radiosensitivity and defective DSB rejoining to that found in 411BR and 180BR (Figures 1 and 5). LB 2304 also displayed sensitivity in an assay that monitors radiosensitivity in LCLs as well as defective

subtracted prior to these calculations. The results represent the mean and standard deviation of at least two gel runs on at least two plug preparations.

(C-E) 411BR cells express normal levels of ligase IV but show impaired adenylation and ligation activities.  $\alpha$ -Xrcc4 immunoprecipitates were examined as follows: (C) by Western blotting using  $\alpha$ -ligase IV, (D) for adenylation activity, and (E) for nick ligation activity. CZD82CH is an NBS cell line, N114-P2 is a *LIG4* null preB cell line, and Nalm6, the preB parental line (Grawunder et al., 1998a). Experiments (C)-(E) have been carried out three times.

Table 1. Clinical Features of DNA Ligase IV-Deficient Patients

Features	Patient 411BR	Patient 2303	Patient 2304	Patient 99P0149
Age	9	46	48	9
Facial features	"bird-like"	Seckel-like	Seckel-like	Seckel-like
Microcephaly	microcephaly at birth, not evident at age 9	microcephaly	microcephaly	microcephaly
Developmental/growth delay	global developmental delay	growth retardation	growth retardation	primordial dwarfism, developmental/mental delay
Immunodeficiency	pancytopenia (cytopenia, lymphopenia, thrombocytopenia)	pancytopenia, myelodysplasia, sinusitis	chronic respiratory infections	pancytopenia
Skin conditions	extensive plantar warts	chronic skin conditions, photosensitivity, telangiectasia	photosensitivity, psoriasis	multiple psoriasiform erythrodermic, squamous skin patches
Cancer	none	none	none	none
Other		hypothyroid, type II diabetes, hypogonadism	hypothyroid, amenorrhea	atypical bone maturation
<i>LIG4</i> genotype	8C>T (A3V); 26C>T (T9I); 833G>A (R278H) all changes homozygous	1738C/T (R/X580); 2440C/T (R/X814) compound heterozygote	1738C/T (R/X580); 2440C/T (R/X814) compound heterozygote	2440C/T (R/X814); 1406G/A (G/E469) compound heterozygote

DSB rejoining (data not shown) (Huo et al., 1994). A cell line has not been derived from patient 99P0149.

Residual DNA ligase IV protein was not detectable in extracts from FB2303 or LB2304 by Western blotting or following immunoprecipitation with either anti-ligase IV or anti-Xrcc4 (Figure 2A). As described below, the larger, although not the smaller, truncated protein is recognized by the  $\alpha$ -ligase IV antibody used here. Anti-Xrcc4 immunoprecipitates were also examined in adenylation and ligation assays. As anticipated from the lack of detectable DNA ligase IV, no residual activity in either assay was detected (Figures 2B and 2C).

By immunofluorescence, DNA ligase IV was localized primarily to the nucleus in 1BR3 and 411BR cells although cytoplasmic staining was also evident (Figure 3). Similar results were obtained using 180BR cells (data not shown). Thus, the combined A3V, T9I, and R278H mutations do not significantly impair the stability or localization of ligase IV. Markedly reduced levels of ligase IV were observed in LB2304 cells although the signal was correctly localized to the nucleus. The level of signal in LB2304 cells was slightly greater than that observed in the *LIG4* null cell line, N114-P2. We conclude that the stability of the truncated proteins in LB2304 is dramatically decreased although the studies suggest that a low level of residual protein is expressed.

#### Analysis of the Impact of the *LIG4* Mutations Using Recombinant Mutant Protein

To examine the impact of the mutations on ligase IV function, the mutations were introduced into *LIG4* cDNA by site-directed mutagenesis, and the proteins were coexpressed with wild-type (WT) Xrcc4 using rabbit reticulocyte lysates to derive WT or mutant ligase IV/Xrcc4 complexes. To examine the interaction with Xrcc4, radiolabeled proteins were expressed and the presence of ligase IV was examined in anti-Xrcc4 immunoprecipitates. The activity of the recombinant complexes was examined using a nick ligation assay.

Recombinant complexes containing the R278H muta-

tion interacted with Xrcc4 as efficiently as WT complexes (Figure 4A, lane 6). In the nick ligation assay, the R278H mutant complex gave background activity similar to lysates prepared from empty vector controls (Figure 4B, lanes 3 and 4) in contrast to the significant ligation activity obtained with WT complexes (Figure 4B, lane 2). We also examined whether the two N-terminal amino acid substitutions, A3V and T9I, present in 411BR but absent in 180BR, affect ligase IV function alone or enhance the impact of the R278H mutation. Complexes containing the N-terminal substitutions, with or without the R278H mutation, showed the same ability to interact with Xrcc4 (Figure 4A, lanes 4 and 5). Complexes with the N-terminal substitutions alone were also as efficient for nick ligation as the WT complex (Figure 4B, lane 1) whereas no activity above background was detected with complexes harboring the combined A3V, T9I, and R278H alterations (Figure 4B, lane 5).

To examine the R580X and R814X mutations, we first expressed radiolabeled mutant ligase IV in the absence of Xrcc4. Truncated proteins of the predicted sizes (80 and 60 kDa) were detected (Figure 4C). The R814X but not R580X protein was recognized by the anti-ligase IV antibody (SJA4), which was raised to a C-terminal fragment of ligase IV (Figure 4B). To investigate the ability of the R814X protein to interact with Xrcc4, recombinant complexes derived by coexpression with Xrcc4 were immunoprecipitated using anti-Xrcc4. A dramatically reduced level of an 80 kDa protein was found in the anti-Xrcc4 immunoprecipitates obtained using R814X mutant complexes in contrast to the signal obtained with WT complexes (Figure 4D). We conclude that R814X truncation markedly impairs interaction between Xrcc4 and DNA ligase IV. Since the R580X protein has a similar mobility to Xrcc4, we did not assess its ability to interact with Xrcc4.

Finally, we examined the R580X and R814X mutant complexes for nick ligation activity. In contrast to the WT complex, no activity above background could be detected with either complex (Figure 4B, lanes 5 and 7). DNA ligase IV expressed in the absence of Xrcc4 did

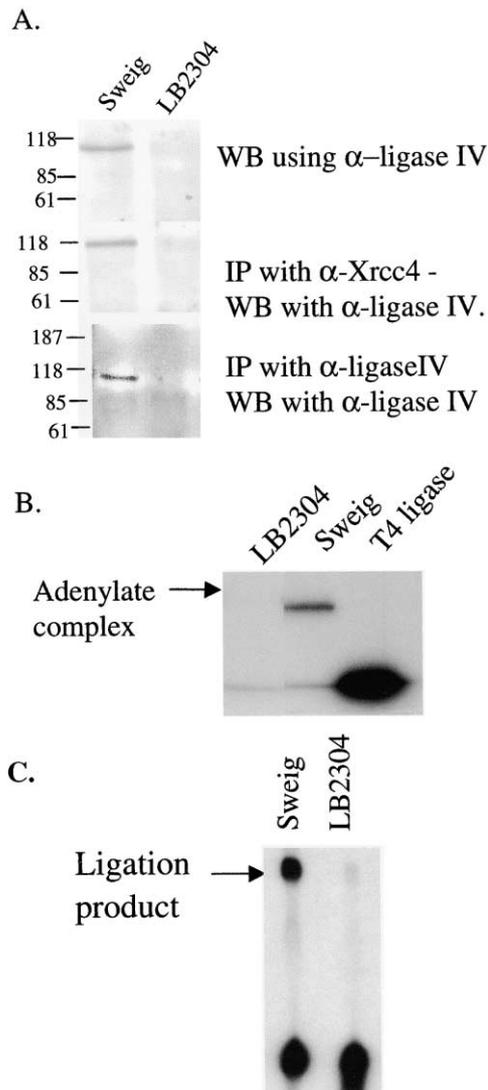


Figure 2. Cellular Analysis of LB2304 Cells

(A) DNA ligase IV is undetectable in LB2304 by Western blotting. Cell extracts from LB2304 cells were examined for expression of DNA ligase IV by Western blotting using  $\alpha$ -ligase IV directly (top panel) or following immunoprecipitation with  $\alpha$ -Xrcc4 or  $\alpha$ -ligase IV (middle and bottom panels, respectively). No residual protein could be detected in LB2304 cells in contrast to results obtained with extracts from Sweig, a control LCL.

(B and C) LB2304 cell extracts have undetectable adenylation and ligation activity following  $\alpha$ -Xrcc4 immunoprecipitation.

not show nick ligation activity (Figure 4B, lane 9). Since the R814X truncation does not affect the ligase domain of *LIG4*, it is likely that the lack of nick ligation activity of the R814X protein results from impaired interaction with Xrcc4.

The impact of the G469E mutation, found in patient 99P0149, was similarly examined. The G469E mutant protein retained its ability to interact with Xrcc4 (Figure 4A, lane 7), but no ligation activity above background was observed (Figure 4B, lane 8). We conclude that the G469E mutation, which is located within the ligase domain, impairs ligase IV function but not its interaction with Xrcc4.

#### Expression of *LIG4* cDNA Corrects the Defects in 411BR and FB2303 Cells

To verify that defects in DNA ligase IV are responsible for the phenotypes described here, we used retroviral transduction to introduce *LIG4* cDNA into FB2303 and 411BR primary fibroblasts. FB2303 cells infected with the *LIG4* retrovirus, but not vector alone, expressed a similar level of full-length ligase IV to that observed in a control fibroblast cell line by Western blotting (Figure 5A). *LIG4*-infected FB2303 cells were fully corrected for radioresistance and DSB rejoining (Figures 5B and 5C). The nick ligation and adenylation activities of *LIG4*-infected FB2303 fibroblasts also returned to levels observed in control cells (data not shown). Infection of 411BR cells with the *LIG4* retrovirus also restored expression of DNA ligase IV and normal radioresistance (data not shown).

#### LB2304 and 411BR Cells Have a Unique Defect in V(D)J Recombination

The frequency and fidelity of V(D)J recombination was examined in 411BRneo and LB2304 cells. The frequency of signal and coding join formation in 411BRneo cells was only marginally reduced compared to that obtained in control 1BR3neo cells but could not be assessed in LB2304 cells due to poor transfection efficiency (Figure 6A). Strikingly, we observed a significant change in signal join fidelity from 98% precision in 1BR3neo cells to less than 40% in 411BRneo and LB2304 cells (Figure 6A). Ten signal junctions sequenced from 411BR had deletions ranging from 18–35 with an average of 26 bp. We also observed a modest increase in the average size of deletions at 10 coding junctions sequenced from 411BRneo cells (7 to 27 nucleotides) compared to 1BR3neo cells (1 to 8 nucleotides).

#### Increased Spontaneous Chromosomal Breakage in Patient 2303

The analysis of chromosome breakage in lymphocytes has played an important role in diagnosis of A-T and NBS. Primary peripheral blood lymphocytes (PBLs) from patient 2303 showed a 10-fold increase in spontaneous chromosome breakage compared to control lymphocytes (22% of metaphases had chromosome breaks). Neither patient 99P0149 nor 411BR showed reproducible increased breakage although lymphopenia precluded detailed analysis. No translocations or inversions involving chromosomes 7 and 14, which are characteristic of A-T and NBS, were observed in PBLs from any of the *LIG4* syndrome patients. Chromosome breaks were significantly elevated in 411BR fibroblasts in response to 1.0 Gy radiation (1.98 breaks/cell in 411BR compared to 0.44 breaks/cell for 1BR3;  $\chi^2 = 47.93$ ; 1 df;  $P < 0.001$ ) and in 99P0149 lymphocytes in response to bleomycin (data not shown) confirming that mutations in *LIG4* confer chromosomal radiosensitivity.

#### Ligase IV Defective Lines Form Spontaneous and Radiation-Induced Nibrin-Dependent Foci and Have Functional Cell Cycle Checkpoints

The clinical overlap between these *LIG4* syndrome and NBS patients prompted us to examine the *LIG4*-defective cells for phenotypes known to be impaired in NBS

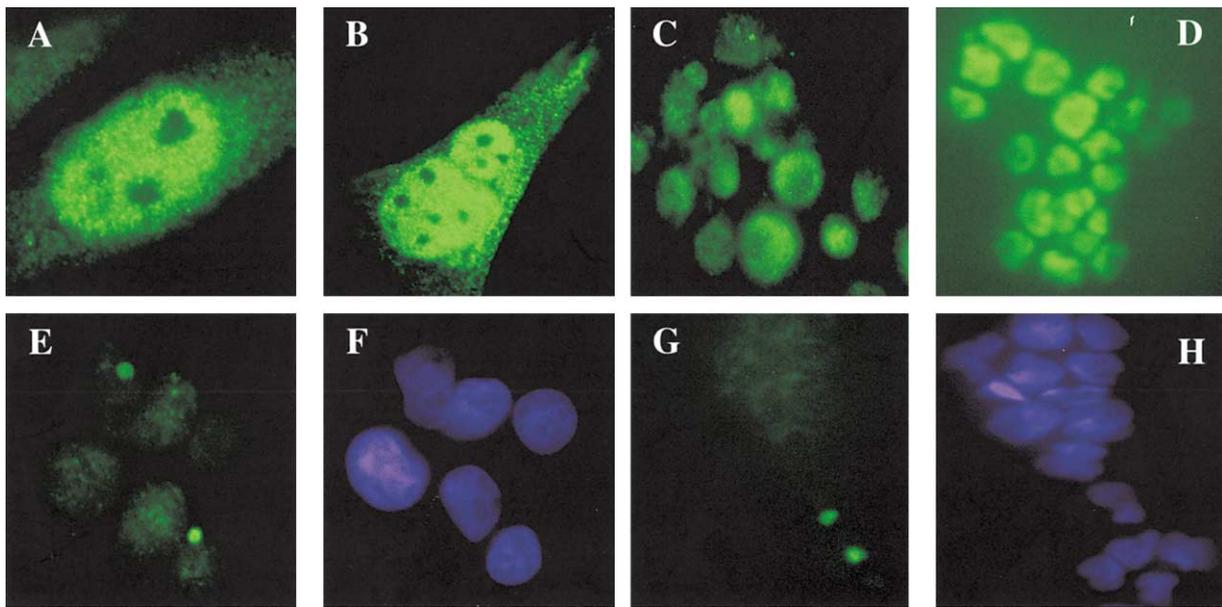


Figure 3. Immunolocalization of DNA Ligase IV

Cells were stained for immunofluorescence using  $\alpha$ -ligase IV (SJ4A) or for DNA using DAPI. Panels are as follows: (A) 1BR3; (B) 411BR; (C) Sweig (control LCL); (D) Nalm6 cells (control preB line); (E) LB2304; (F) same cells as in (E), stained with DAPI; (G) *LIG4* null line (N114-P2); and (H) same cells as in (G), stained with DAPI. No change in immunolocalization was seen in control or mutant cell lines following exposure to IR (data not shown).

cell lines. First, we analyzed hMre11/hRad50/nibrin foci formation in 411BR and FB2303 fibroblasts. We observed normal nuclear localization of hMre11 and nibrin in the ligase IV-deficient fibroblasts (data not shown) and normal induction of foci following exposure to 12 Gy  $\gamma$ -rays (Figure 6B).

Next, we examined the operation of the G1/S, S, and G2/M checkpoints in primary 411BR cells. 411BR showed a normal DNA synthesis arrest following exposure to radiation (Figure 5C) in contrast to the typical defect observed in A-T and NBS cell lines, providing a simple assay to distinguish *LIG4* syndrome from NBS patients. 411BR also arrested normally at the G1/S checkpoint (Figure 5D). p53 stabilization and p21 induction also occurred normally in 411BR cells (data not shown). Finally, control cells in G2 at the time of irradiation show a delay in entry into mitosis postirradiation, representing an ATM-dependent G2/M checkpoint. 411BR cells show a substantial delay in entry into mitosis following exposure to 2 Gy similar to that shown by control cells (Figure 5E). Taken together, these findings demonstrate that 411BR shows normal radiation-induced cell cycle checkpoint arrest.

## Discussion

Here we describe patients with defects in DNA ligase IV, a protein that functions in DNA NHEJ. The clinical features of the patients resemble those of NBS but the cellular phenotype is distinct. Cell lines from both *LIG4* and NBS patients show radiosensitivity but, in contrast to NBS lines, *LIG4* cell lines are defective in DSB repair and have normal checkpoint function. These findings represent the identification of an additional protein con-

tributing to primary human immunodeficiency and define an inherited disorder arising from a specific DNA repair defect.

A homozygous mutation in *LIG4* has been previously described in a developmentally normal leukemia patient (180BR) (Riballo et al., 1999). Patients 411BR, described here, and 180BR share the same homozygous mutation, 833G>A, which results in the substitution, R278H, within the ligase IV active site, significantly impairing ligase IV function (Riballo et al., 2001). One explanation for the marked difference in clinical features is that patient 180BR may have displayed an unrecorded partial phenotype. Unfortunately, a retrospective examination of the patient's phenotype is precluded since his clinical records are no longer available. It is also possible that other genetic differences contribute to the clinical characteristics. An alternative explanation, however, is suggested by the two homozygous N-terminal substitutions (A3V and T9I) present in 411BR but not in 180BR. These represent polymorphic changes since they have been observed in normal individuals (P. Roddam, personal communication). The N-terminal region of ligase IV is not highly conserved although it is essential for function (Grawunder et al., 1998b). Neither of these substitutions is likely to have a dramatic impact on conformation although the combined mutations will increase the hydrophobic nature of this region of the protein. Our in vitro studies demonstrate that the two substitutions alone do not grossly impair ligase IV activity, but an impact upon the residual activity of the R278H mutant protein cannot be ruled out. Such an effect, even if modest, in a patient with already significantly impaired ligase IV activity could be sufficient to cause the more overt phenotype reported for patient 411BR. Indeed, an examination of

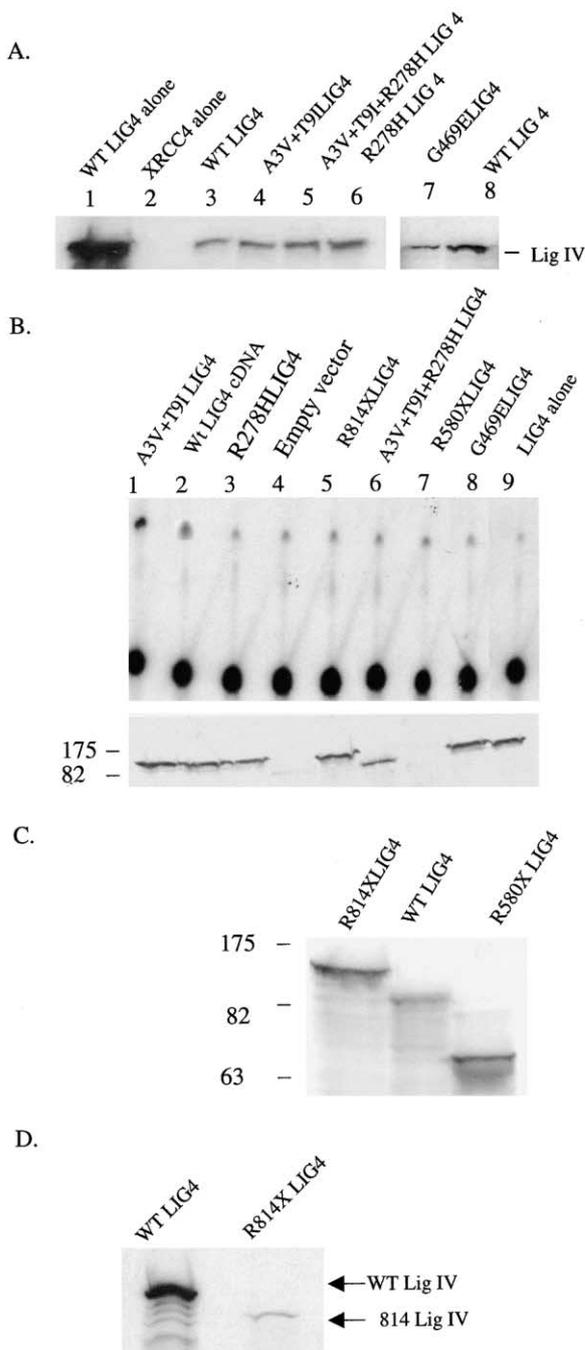


Figure 4. Analysis of Recombinant Proteins Expressing DNA Ligase IV Mutations

Recombinant wild-type (WT) or mutant ligase IV was expressed in rabbit reticulocyte lysates with WT Xrcc4 unless stated otherwise. Mutant ligase IV proteins carrying the amino acid changes indicated were generated by site-directed mutagenesis of WT *LIG4* cDNA using the base substitutions identified in the patients.

(A) Interaction of mutant ligase IV with Xrcc4. Proteins were expressed in the presence of <sup>35</sup>S-methionine, immunoprecipitated using  $\alpha$ -Xrcc4 antibodies, and size fractionated by SDS-PAGE gel electrophoresis and subjected to autoradiography. Lanes 1 and 2 represent WT ligase IV or Xrcc4, respectively, expressed alone; the remaining lanes are as indicated and were coexpressed with Xrcc4. Only the ligase IV band has been shown although Xrcc4 was also radiolabeled.

Table 1 demonstrates that there is substantial phenotypic variation between the LIG4 syndrome patients that is likely related to the nature of the mutations.

Since *LIG4* null mutations in mice result in embryonic lethality, it is likely that the mutations described here are hypomorphic (Barnes et al., 1998; Gao et al., 1998). Two of the mutations identified lead to protein truncations resulting in deletion of both Brct domains or the second Brct domain in addition to part of the intervening region between the two domains. The intervening region has been reported to be important for interaction with Xrcc4 (Grawunder et al., 1998b). Consistent with this, the R814X mutation impairs interaction with Xrcc4. Only low residual ligase IV protein was detectable in cell lines expressing the two truncating mutations. Two factors, therefore, contribute to the defect in these patients: decreased levels of ligase IV and impaired interaction with Xrcc4. The fourth mutation, G469E, lies within the ligase domain. G469 is located on a surface loop in a region of DNA ligase IV important for DNA binding. Based on the crystal structure of T7 ligase, this loop faces away from the active site cleft but is on the proposed DNA binding face of the protein. Following adenylation, this face rotates to facilitate DNA binding. The G to E substitution, replacing a small, uncharged amino acid with a bulky negatively charged residue, is predicted to disrupt the structure of the loop. Taken together, the mutations identified here suggest that either disrupting the interaction of ligase IV with Xrcc4 or directly impairing ligase IV activity can confer a LIG4 syndrome phenotype.

Several of the LIG4 patients are compound heterozygotes, raising the possibility that *LIG4* mutations may be more frequent than suggested by the limited collection of patients identified here. Many of the features of LIG4 syndrome patients resemble those of Seckel syndrome, a form of dwarfism. Indeed, a subset of Seckel syndrome patients show chromosome instability, pancytopenia, and adverse responses to chemotherapy (Butler et al., 1987). Based on our findings, we recommend screening of further NBS-like and Seckel syndrome patients for defects in NHEJ components and an analysis of the relationship between NHEJ and syndromes characterized by dwarfism.

(B) Ligation activity of recombinant mutant ligase IV/Xrcc4 complexes. Lanes 1–8, WT or mutant ligase IV was coexpressed with Xrcc4 and subjected to nick ligation without prior immunoprecipitation; lane 9, WT ligase IV was expressed in the absence of Xrcc4. The lower panel shows the in vitro expression level of ligase IV. The R580X protein is not recognized by the  $\alpha$ -ligase IV antibody used. Its expression was verified using <sup>35</sup>S-methionine (C). Similar results have been obtained in three separate experiments.

(C) The 1738C>T and 2440C>T mutations result in truncated ligase IV. Mutant and WT ligase IV was expressed in the absence of Xrcc4 but presence of <sup>35</sup>S-methionine and size fractionated by SDS-Page gel electrophoresis. Both the R814X and R580X mutations result in the expression of truncated proteins of the predicted size (80 and 65 kDa, respectively).

(D) R814X ligase IV has reduced ability to interact with Xrcc4. WT or mutant ligase IV was coexpressed with Xrcc4 in the presence of <sup>35</sup>S-methionine, and  $\alpha$ -Xrcc4 immunoprecipitates were analyzed by SDS-page electrophoresis. While similar levels of WT and R814X ligase IV proteins were expressed (data not shown but results similar to [C]), dramatically decreased levels of R814X protein were present in the immunoprecipitates.

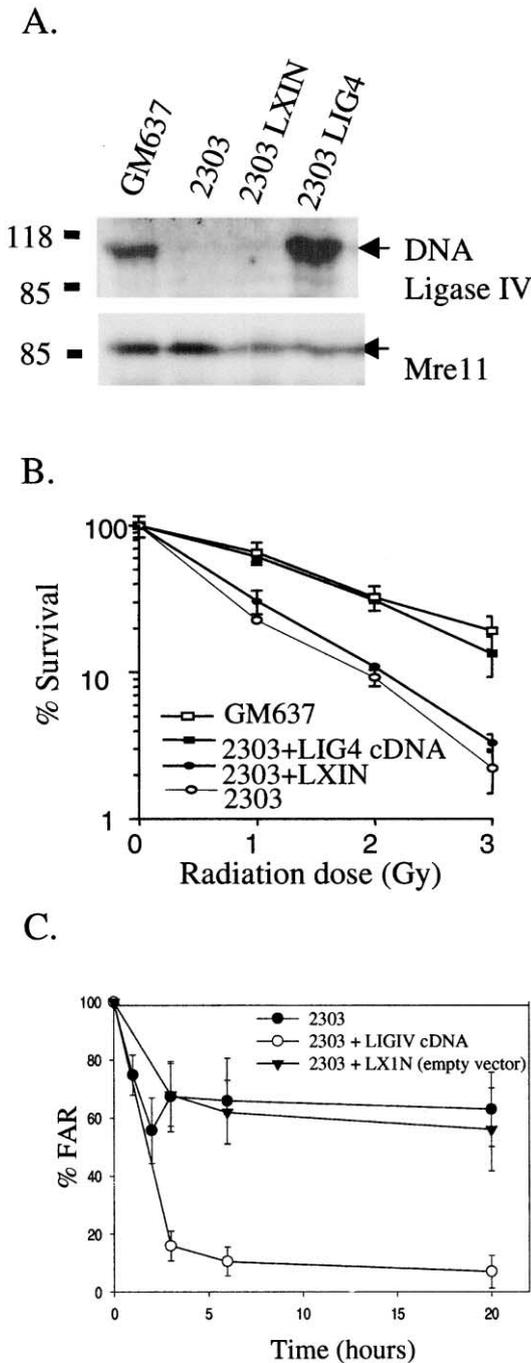


Figure 5. FB2303 Cells Are Complemented by Expression of *LIG4* cDNA

Primary FB2303 cells were infected with supernatants from empty retroviruses (pLXIN) or from retroviruses expressing *LIG4* cDNA and analyzed for (A) expression of ligase IV by Western blotting with  $\alpha$ -ligase IV (B) survival and (C) DSB rejoining following exposure to IR. GM637 is a primary fibroblast line from a normal individual.

Given the strong evidence that ligase IV functions in V(D)J recombination, it is surprising that we observed only a marginal decrease in V(D)J recombination frequency in 411BR. The *LIG4* null line, N114-P2, did show a marked defect in the plasmid assay, attesting to its suitability to monitor V(D)J recombination (Grawunder et al., 1998a). This suggests that a low level of residual

activity is sufficient to effect rearrangements in this assay. We did observe, however, decreased signal joint fidelity conferred by impaired ligase IV activity coupled with a modest increase in the size of coding joint deletions. Such an impairment of signal joint fidelity has also recently been reported for 180BR (Riballo et al., 2001). The *LIG4* patients displayed pancytopenia but not a SCID phenotype consistent with the notion that the mutations also have a partial impact on V(D)J recombination in vivo.

The similarity of clinical features between NBS and *LIG4* syndrome raises the question of the role of nibrin in NHEJ. The Mre11/Rad50/Xrs2 complex is required for NHEJ in the yeast *S. cerevisiae*, but not in *S. pombe* (Manolis et al., 2001). NBS cells are proficient for V(D)J recombination and DSB repair, hallmarks of defective NHEJ (Girard et al., 2000; Yeo et al., 2000). Conversely, the *LIG4* cell lines described here with mutations sufficiently severe to cause developmental delay have functional checkpoints, a hallmark of NBS. The pronounced radiosensitivity of A-T and NBS cell lines has been attributed to a repair defect separable from the checkpoint defect, although the underlying basis is unknown and both appear proficient in HR as well as NHEJ (Jeggo et al., 1998). It is likely that some clinical features of A-T and NBS may be attributed to the repair defect, others to the checkpoint phenotype, and some to the dual defect (Jeggo et al., 1998). The similar features of NBS and *LIG4* syndrome could be general characteristics displayed in common by patients lacking damage response mechanisms. *LIG4* is essential for embryonic development and null mice display elevated apoptosis in developing neurones. Mice with inactivating mutations in several other damage response genes display a similar phenotype of impaired embryonic development and neuronal apoptosis (Deans et al., 2000). This raises the exciting possibility that our findings have broader significance and that defects in other damage response genes may be found within the group of NBS-like and Seckel-like patients. In this context, a patient defective in DNA ligase I also displayed growth delay and immunodeficiency (Barnes et al., 1992). Alternatively, it is possible that nibrin, although not a core NHEJ component, may have an impact upon the same overall repair pathway. Finally, since the phenotype of *LIG4* syndrome is due to defective repair, this raises the possibility that the developmental phenotype in NBS may also be attributable to the repair rather than the checkpoint defect.

An important characteristic of A-T and NBS is elevated cancer incidence, raising the question as to whether *LIG4* syndrome is a cancer predisposition disorder. The increased cancer incidence in A-T and NBS patients likely reflects their dual checkpoint/repair defects. Since *LIG4* syndrome cells maintain a normal checkpoint response, it is possible that any aberrant cells arising from the repair defect may undergo cell death rather than proliferation. Currently, there are too few patients of sufficient age to allow any firm conclusions to be drawn. While patient 180BR developed leukemia, patients 2303 and 2304, at ages 46 and 48, respectively, are older than most A-T and NBS patients and have not developed cancer.

In conclusion, we have described a syndrome resulting from a defect in NHEJ due to mutations in the gene encoding DNA ligase IV. This demonstrates that a

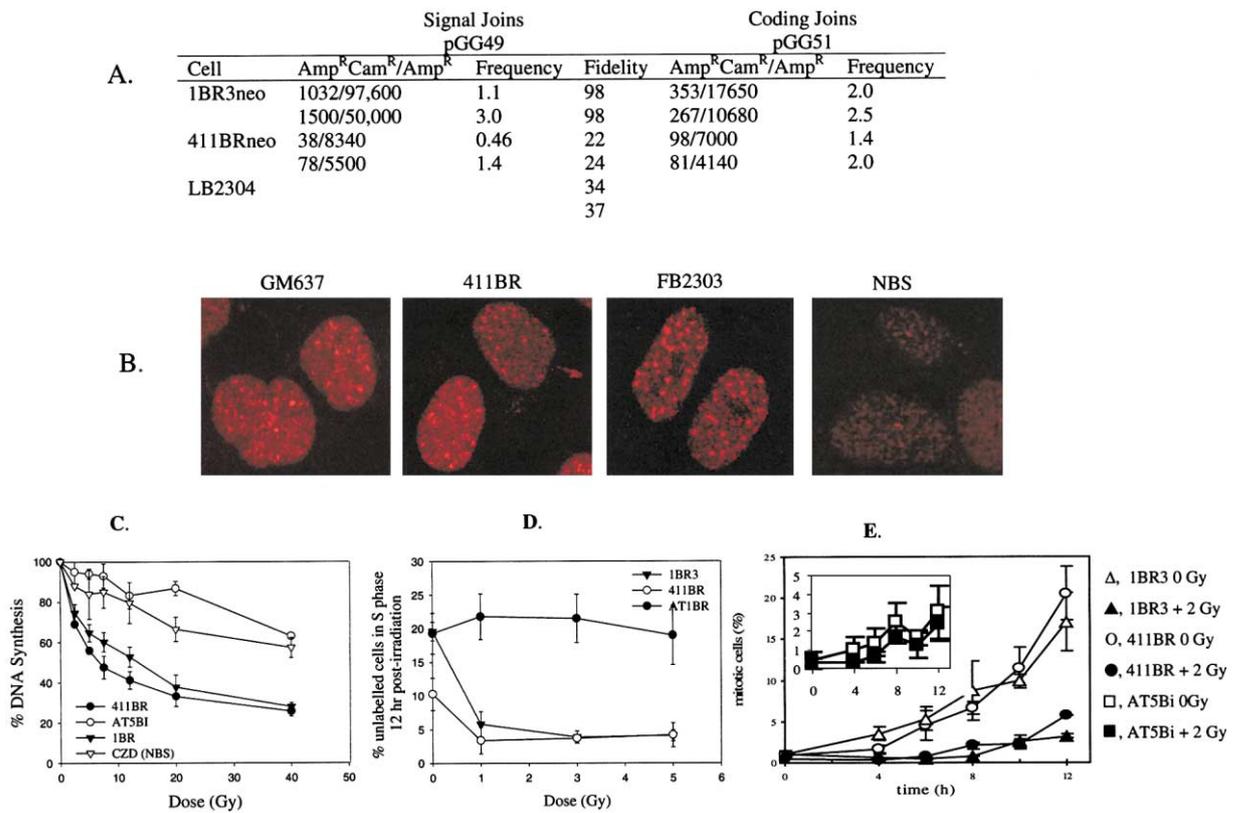


Figure 6. V(D)J Recombination, Radiation-Induced Nibrin Foci Formation, and Cell Cycle Checkpoint Analysis in DNA Ligase IV Defective Cell Lines

(A) The frequency and fidelity of V(D)J recombination in *LIG4* defective cell lines. Results shown represent two independent experiments. V(D)J recombination frequencies were not estimated with the FB2304 cell line due to insufficient numbers of transfectants.

(B) Nibrin radiation induced foci formation in *LIG4*-deficient primary fibroblasts, 411BR, FB2303, and an NBS line, were exposed to 12 Gy of IR and examined for nibrin foci formation after 7 hr.

(C) 411BR cells show normal S phase checkpoint arrest. 411BR, AT5BI, and CZD82CH, an NBS cell line, were examined using the RDS assay to monitor S phase checkpoint arrest. The results shown represent the % DNA synthesis relative to unirradiated cells following exposure to the radiation doses indicated.

(D) 411BR cells show normal G1/S arrest. S phase cells are labeled by a 30 min exposure to BudR immediately prior to irradiation. Following FACS, the progression of unlabeled cells (i.e., those that have not traversed the G1/S boundary at the time of irradiation) into S phase is estimated. The results shown represent the mean and standard deviation of three experiments for all lines.

(E) 411Br cells show normal G2/M arrest. The accumulation of mitotic cells in the presence of the mitotic inhibitor, colcemid, was scored in untreated cells (open symbols) or following exposure to 2 Gy (closed symbols).  $\Delta$  and  $\square$  represent 411BR and 1BR3, respectively. The inset panel shows the results with AT5BI. Note the different scale for these cells, which have a lower mitotic index. At least 1000 cells were scored for each time. Results shown represent the mean and SD of three experiments.

defect in DNA NHEJ can contribute to a clinical disorder associated with immunodeficiency and developmental delay.

#### Experimental Procedures

##### Cell Culture Methods

Primary skin fibroblast cultures and LCLs were derived using standard procedures. 411BRneo is an SV40-transformed cell line derived from primary 411BR cells; 1BR3neo is an SV40-transformed and immortalized control cell line. Cells were cultured in Minimal Essential Medium (MEM) and analyzed for radiosensitivity as described previously (Arlett et al., 1988). CZD82CH, an NBS line and N114-P2, a *LIG4* null pre-B cell line, were kindly provided by Drs. J. Hall and K. Chrzanowska, and Dr. M. Lieber, respectively.

##### Patients' RNA and DNA Samples and Sequence Analysis

Blood samples were obtained from patients under informed consent. DNA was extracted from whole blood using a Nucleon II Kit (Scotlab) and total RNA was extracted from LCLs. Overlapping fragments of the *LIG4* gene were PCR amplified from DNA using primers:

F1 5'-GTAGACTGCGCCGATTAG-3', R1 5'-TCTACATCATTCTCTGCC-3', F2 5'-CTCAGTGATCTTTGAAGATC-3', R2 5'-GTA AACATCTGGCTTCAACAC-3', F3 5'-GGAGATGCTGGAGACTTGC-3', R3 5'-TTATAGGCATCATCTACCA-3', F4 5'-CCATT CATTATAATGCATTTC-3', R4 5'-AAAGGCTTCCAATACTTGGC-3', F5 5'-GGCTGCACCATGAAAGA-3', R5 5'-CATGCAGGCTTGA CAACATC-3', F6 5'-GTACAAATCCAGGCCAGAC-3', R6 5'-CAT GAGACATCCCTCAGC-3', F7 5'-AGCTTCGGTTTCATGGAGC-3', R7 5'-TCTTTCTTGCTTTGGGCTA-3'. Amplicons were sequenced using Big Dye fluoresceinated dye terminators (PE Applied Biosystems) and analyzed on an ABI Prism 377 sequencer.

##### Pulse Field Gel Electrophoresis for Measurement of DSB Repair

The procedure used was as described previously (Riballo et al., 2001).  $2-3 \times 10^6$  cells were labeled with 0.02  $\mu$ Ci/ml <sup>14</sup>C-thymidine (Amersham Life Sciences, 50-60 mCi/mmol) for 48-50 hr followed by incubation for 2 hr in unlabeled medium. Agarose plugs were prepared from the cells, irradiated with 40 Gy  $\gamma$ -rays, and incubated for repair. The cells were lysed in the plugs, and DNA fragments were separated using a Biorad CHEF DRIII. After electrophoresis, the gel was placed onto DE18 paper (Whatman), dried for 3 hr at

50°C, and analyzed on a STORM phosphorimager using ImageQuant software (Molecular Dynamics).

#### Cell Cycle Analysis

G1/S arrest was assayed using FACS as described previously (Badie et al., 1997). Cells were labeled for 30 min with  $10^{-5}$  M 5-Bromo-2'-deoxyuridine (BrdU), washed, and then irradiated or maintained as controls for 6, 12, 18, and 24 hr. Cells were separated by FACS analysis on the basis of DNA content using PI and BrdU incorporation using the FITC signal. Percent G1/S arrest is calculated from the unlabeled G1 cells/total unlabeled cells at 12 hr postirradiation following exposure to 1, 3, or 6 Gy relative to that obtained in control unirradiated cells. The analysis of G2/M checkpoint involved counting the mitotic index as described previously (Girard et al., 2000). The estimation of radioresistant DNA synthesis was performed as described previously (Jaspers, 1985).

#### Immunofluorescence

The procedure, fixation and permeabilization, was carried out essentially as described previously (Scully et al., 1997). LBLs were centrifuged onto slides using a Cytospin at 500 rpm for 5 min. Primary  $\alpha$ -Ligase IV antibody (SJA4) was added for 20 min at 37°C at 1/100 dilution in 2% BSA/PBSA followed by FITC-conjugated anti-rabbit antibody (Sigma) at 1/200 dilution in 2% BSA/PBSA for 20 min. Slides were incubated with DAPI (1:50,000 of 1 mg/ml Stk in PBS) for 10 min at 4°C in the dark.

#### Biochemical Procedures

Preparation of cell free extracts and  $\alpha$ -Xrcc4 immunoprecipitation was carried out essentially as described previously (Riballo et al., 1999). To monitor adenylation, extracts were pretreated with 5 mM di-sodium pyrophosphate for 15 min at room temperature in adenylation buffer (60 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>, and 5 mM DTT).  $\alpha$ -<sup>32</sup>P-ATP (0.5  $\mu$ Ci, ICN) was added to the reaction and incubated for 10 min at room temperature. Reactions were stopped by addition of 1 volume of 2 $\times$  standard SDS-PAGE buffer. The samples were boiled for 5 min and run on a 9% SDS-PAGE gel. The nick ligation assay was performed as described previously (Riballo et al., 2001). The Promega TNT T7 Quick Coupled Transcription/Translation system was used for in vitro transcription/translation. An amino-His-tagged DNA ligase IV cDNA was subcloned in-frame into the expression vector pcDNA3 (Invitrogen) for use in these experiments and for site-directed mutagenesis.

#### Retroviral Transfection

Complementation using retroviral expression vectors was performed as described (Cerosaletti et al., 2000). A full-length cDNA copy was obtained from the IMAGE consortium (clone #547945) and a 3052 bp EcoRI-XbaI fragment was cloned into the HpaI site of pLXIN (Clontech), upstream of the IRES-neomycin cassette. This fragment extended from -32 bp upstream of the first ATG of LIG4 to 287 bp 3' of the stop codon, upstream of the poly A signal. pLXIN without insert was used as a negative control. Retroviral DNA was packaged in Phoenix A cells. Retroviral supernatants were harvested 48 hr after transfection, filtered to remove residual packaging cells, and used to infect 411BR and FB2303 cells. Growth medium was replaced 24 hr after infection. At 48 hr postinfection, cells were placed in fresh medium supplemented with 500  $\mu$ g/ml of G418. Bulk transformed lines were established within 2 weeks.

#### Analysis of V(D)J Recombination

Assays to examine the frequency of V(D)J recombination were performed as described previously using the recombination substrates, pGG49 and pGG51 (Gauss et al., 1998; Gauss and Lieber, 1996). To assess the fidelity of signal joint formation, colonies bearing rearranged pGG49 plasmids were subjected to colony-filter hybridization and challenged with a probe (SJ2) specific for a precise signal joint and the percent of hybridizing clones determined (Riballo et al., 2001).

#### Nuclear Focus Formation

Nibrin radiation-induced focus formation was analyzed as described (Cerosaletti et al., 2000). To detect nibrin, cells were costained with

rabbit anti-p95 antisera (Novus Biologicals), followed by Alexa Fluor 568 goat anti-rabbit IgG conjugate and Alexa Fluor 488 goat anti-mouse IgG conjugate (Molecular Probes). Nuclei were counterstained with TOTO-3 iodide (Molecular Probes). Cellular fluorescence was analyzed using a Nikon fluorescent microscope and a Bio-Rad confocal imaging system.

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#### Note Added in Proof

Chen et al., (*Mol. Cell* 8, 1105–1115; 2001) have recently demonstrated that in *S. cerevisiae*, the Rad50/Mre11/Xrs2 (NBS1) complex interacts directly with the Dnl4/Lif1 (LIG4/XRCC4) complex and promotes the DNA end-ligation activity of Dnl4/Lif1. These findings suggest that the similarities in the phenotypes of NBS and LIG4 patients may reflect the formation of a complex incorporating the products of the genes mutated in these disorders.