

XRCC4 deficiency in human subjects causes a marked neurological phenotype but no overt immunodeficiency

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Background: Nonhomologous end-joining (NHEJ) is the major DNA double-strand break (DSB) repair mechanism in human cells. The final rejoining step requires DNA ligase IV (LIG4) together with the partner proteins X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor. Patients with mutations in genes encoding LIG4, XRCC4-like factor, or the other NHEJ proteins DNA-dependent protein kinase catalytic subunit and Artemis are DSB repair defective and immunodeficient because of the requirement for NHEJ during V(D)J recombination.

Objective: We found a patient displaying microcephaly and progressive ataxia but a normal immune response. We sought to

determine pathogenic mutations and to describe the molecular pathogenesis of the patient.

Methods: We performed next-generation exome sequencing. We evaluated the DSB repair activities and V(D)J recombination capacity of the patient's cells, as well as performing a standard blood immunologic characterization.

Results: We identified causal mutations in the *XRCC4* gene. The patient's cells are radiosensitive and display the most severe DSB repair defect we have encountered using patient-derived cell lines. In marked contrast, a V(D)J recombination plasmid assay revealed that the patient's cells did not display the junction abnormalities that are characteristic of other NHEJ-defective cell lines. The mutant protein can interact efficiently with LIG4 and functions normally in *in vitro* assays and when transiently expressed *in vivo*. However, the mutation makes the protein unstable, and it undergoes proteasome-mediated degradation.

Conclusion: Our findings reveal a novel separation of impact phenotype: there is a pronounced DSB repair defect and marked clinical neurological manifestation but no clinical immunodeficiency. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: DNA double-strand break repair, nonhomologous end-joining, double-strand break repair deficiency, *XRCC4/LIG4*, immunodeficiency, microcephaly

Double-strand breaks (DSBs) arise from oxidative damage and during processes such as meiosis or V(D)J recombination. They are also the major lethal lesions generated in cellular DNA by ionizing radiation. Although DSBs do not arise at high frequency endogenously, they are a critical lesion causing cell death or carcinogenesis if misrepaired. Additionally, the development of the immune response involves introduction of DSBs to generate the requisite genetic diversity.¹ V(D)J recombination represents one such process.² Class-switch recombination (CSR), which involves switching of immunoglobulin isotypes, also involves introduction and rejoining of DSBs.³

Nonhomologous end-joining (NHEJ) is the major DSB repair process in mammalian cells. The process is initiated by the binding of the Ku heterodimer to double-stranded DNA ends, followed by recruitment of the DNA-dependent protein kinase (DNA-PK) catalytic subunit, generating the DNA-PK holoenzyme and activating DNA-PK activity.⁴ DNA-PK has a role in end-tethering and regulates the steps of end-processing. The final step of rejoining involves the recruitment of a ligation complex encompassing DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4), and XRCC4-like factor (XLF). LIG4 and

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Abbreviations used

CID:	Combined immunodeficiency
CS:	Cockayne syndrome
CSR:	Class-switch recombination
DNA-PK:	DNA-dependent protein kinase
DSB:	DNA double-strand break
GST:	Glutathione S-transferase
IR:	Ionizing radiation
LIG4:	DNA ligase 4
3-MA:	3-Methyladenine
NHEJ:	Nonhomologous end-joining
NMD:	Nonsense-mediated mRNA decay
RAG:	Recombination-activating gene
SCID:	Severe combined immunodeficiency
WT:	Wild-type
XLF:	XRCC4-like factor
XRCC4:	X-ray repair cross-complementing protein 4

XRCC4 interact strongly through a tandem BRCA1 carboxyl terminal domain in LIG4 and a coiled-coil region in XRCC4, forming a highly stable complex.^{5,6} XRCC4 is required for LIG4 stabilization, but recent findings have shown an excess of XRCC4 relative to LIG4.⁷ Mutations that abolish LIG4/XRCC4 interaction prevent all NHEJ activity. XLF is a weaker binding partner. XRCC4 and XLF are structurally similar; both have a globular head domain, but the coiled-coil region of XLF is shorter than that of XRCC4.^{8,9} Recent studies have reported that dimers of XRCC4 and XLF interact to form long superhelical filaments in solution, which have been proposed to promote end-tethering or end-bridging downstream of the DNA-PK complex.^{8,10-12}

Loss of LIG4 or XRCC4 confers embryonic lethality in mice because of extensive neuronal apoptosis.^{13,14} In contrast, XLF-null mice are viable and do not show marked immunodeficiency.¹⁵ Mutations in *LIG4*, *DCLRE1C* (Artemis), *NHEJ1* (XLF), and *PRKDC* (DNA-PK catalytic subunit) have been described.¹⁶⁻²⁰ LIG4 syndrome, which is caused by hypomorphic mutations in *LIG4*, is characterized by growth delay, microcephaly, and combined immunodeficiency (CID) or severe combined immunodeficiency (SCID). LIG4 syndrome cell lines show radiosensitivity, diminished ability to repair DSBs, and impaired V(D)J recombination. XLF-defective patients, some of whom have null mutations, display features similar to but often more severe than those of patients with LIG4 syndrome.¹⁸ Given the close interaction and requirement of XRCC4 for LIG4 stability, the expectation was that XRCC4 deficiency in patients would resemble LIG4 syndrome.

In contrast to this expectation, an XRCC4-deficient patient, CSL16NG, whose cellular defects we describe in this article, displayed unique clinical features similar to those of patients with Cockayne syndrome (CS), namely progressive neuronal degeneration, including onset of ataxia, but no clinical immunodeficiency. Our cellular analysis revealed that the mutational changes result in substantially reduced levels of XRCC4 protein, a marked defect in DSB repair, but seemingly efficient V(D)J recombination. Based on comparative studies with LIG4- and XLF-deficient cell lines, our results reveal a separation of impact phenotype in which marked deficiency in radiation-induced DSB repair capacity can be uncoupled from defective V(D)J recombination. Our results provide novel insight into the roles of XRCC4 during DSB repair.

METHODS**Human studies**

Patient and control samples were obtained with local ethical approvals (the University of Sussex Research Ethics Committee; the Nagasaki University Ethical, Legal and Social Implications (ELSI) Committee; and the Ethics Committee for Human Genome Studies in Research Institute of Environmental Medicine, Nagoya University). Written informed consent was obtained from the patient.

Exome sequencing and bioinformatics

Details are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Briefly, genomic DNA prepared from CSL16NG fibroblasts was enriched by using the SureSelect All Exon Kit v5 (Agilent Technologies, Santa Clara, Calif), followed by sequencing on the Illumina Hi-Seq 2500 Sequencer (Illumina, San Diego, Calif). The sequence data were analyzed by using a standard exome pipeline. According to a recessive inheritance model, we selected genes that carried at least 1 novel deleterious homozygous or more than 2 heterozygous changes in 1 gene locus (see [Table E1](#) in this article's Online Repository at www.jacionline.org).

Cell cultures

Human primary or human telomerase reverse transcriptase-immortalized fibroblasts were derived from the patient with XRCC4 deficiency (CSL16NG), patients with LIG4 syndrome (F07/614, 180BR, 411BR, 2303, and 495GOS), patients with XLF deficiency (2BN and F07/402), a patient with xeroderma pigmentosum (XP15BR), or healthy donors (1BR.3 and 48BR). All primary fibroblasts were cultured in Dulbecco modified Eagle medium or modified Eagle medium supplemented with 10% to 15% FBS and 1× penicillin-streptomycin. HEK293FT cells (Invitrogen, Carlsbad, Calif) were grown in Dulbecco modified Eagle medium supplemented with 10% FBS, 1× penicillin-streptomycin, 5 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate. Human colon cancer HCT116 (RCB2979) and HCT116-XRCC4^{-/-} (RCB2981) cells were provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Reagents and antibodies

Antibodies used were as follows: XRCC4 (C4 and C20; Santa Cruz Biotechnology, Dallas, Tex), LIG4 (D8; Santa Cruz Biotechnology; Ab80514, Abcam, Cambridge, United Kingdom; 12695-1-AP, Proteintech Group, Chicago, Ill), p89 (S-19, Santa Cruz Biotechnology), XPG (8H7, Santa Cruz Biotechnology), CSB (Bio Matrix Research, Chiba, Japan), V5-tag (MBL, Woburn, Mass), Flag-tag (MBL), actin (Ab8227, Abcam), α -tubulin (Invitrogen), XLF (ab33499, Abcam; FL-299, Santa Cruz Biotechnology), 53BP1 (Bethyl Laboratories, Montgomery, Tex), and γ H2A.X (ser139, Millipore, Temecula, Calif). MG132, E64D, 3-methyladenine (3-MA), epoxomicin, and cycloheximide (Sigma-Aldrich, St Louis, Mo).

Quantitative RT-PCR

Details are as described previously.²¹ Total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, Calif) was used for first-strand synthesis. Quantitative PCR was performed by using the Thermal Cycler Dice Real-Time system (TaKaRa Bio, Clontech, Mountain View, Calif) with a QuantiTect SYBR Green PCR Kit (Qiagen). For each sample, relative mRNA expression levels were normalized by using the hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene. Error bars represent the SDs of means of triplicate experiments.

Lentivirus experiments

Details are as described previously.²² Recombinant lentivirus particles expressing XRCC4 and its mutants were produced. Human XRCC4 cDNA was

cloned in frame with a C-terminal V5-tag in the *pLenti6.3-V5-D-TOPO* vector to generate *pLenti6.3-XRCC4-V5* plasmid. The truncation mutant (XRCC4-Del), the frameshift mutant (XRCC4-Fs), and the single amino acid substitution mutant (XRCC4-W43R) were generated from *pLenti6.3-XRCC4-V5* by means of site-directed PCR mutagenesis. Sequences of the mutant plasmids were confirmed by using Sanger sequencing (see Fig E1 in this article's Online Repository at www.jacionline.org). Forty-eight hours after lentivirus infection, 10 $\mu\text{g/mL}$ blasticidin (Sigma-Aldrich) was added to the medium for selection of XRCC4 stably expressing cells.

Ionizing irradiation and the γH2AX foci assay

Cells were seeded in 3-cm dishes or in 96-well plates (BD Falcon, BD Biosciences, San Jose, Calif) and 3-Gy γ -ray irradiated by using a ^{137}Cs γ -ray source at a dose rate of 0.017 Gy s^{-1} and then further cultured for indicated time periods. Cells were fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100, followed by staining with 4'-6-diamidino-2-phenylindole dihydrochloride and γH2AX antibody. Image acquisition and analysis were performed by using the Cellomics ArrayScan VTI (Thermo Scientific, Waltham, Mass). The average number of separate γH2AX foci was assessed on at least 300 cells.

NHEJ, V(D)J, and CSR assays

The assays were performed as previously described.^{23,24} For the NHEJ assay, 1 μg of PDVG94 plasmid digested with *EcoRV* and *Eco47III* (Promega, Madison, Wis) to create blunt ends with 6-bp repeats were transiently transfected into fibroblast cells by using Turbofect (Fermentas, Vilnius, Lithuania). Forty-eight hours after transfection, cells were harvested and DNA was extracted by using the DNeasy Blood and Tissue Kit (Qiagen). The recombinant junctions were PCR amplified with the primers FM30 and DAR5,²³ and the PCR product was gel purified with the QIAquick gel extraction kit (Qiagen), cloned into pGEM-T vectors, and sequenced (Macrogen, Rockville, Md). The V(D)J assay was performed as the NHEJ assay, except that in total 1.5 μg of PDVG93 circular plasmid and recombination-activating gene (RAG) 1 and RAG2 plasmids were transiently transfected into fibroblast cells and the coding junctions were amplified by a nested PCR with the primers NV05F (5'-CTATAGGGGAATTGTGAGCGGATAACDG-3'), DG147 and DG89, and FM30.²³ Switch recombination junctions were PCR amplified, cloned, sequenced, and analyzed, as described previously.^{25,26}

Protein purification

Detailed protein purification procedures and buffer conditions are as described previously.^{21,27} Briefly, HEK293FT cells were cotransfected with *pLenti6.3-LIG4-His* and *pLenti6.3-Flag-XRCC4* or its mutants. Cell lysates were incubated with anti-Flag M2 beads (Sigma-Aldrich) for 4 hours at 4°C. After extensive washing of the beads, the Flag-tagged wild-type (WT) or mutant XRCC4 in complex with LIG4 was eluted with Flag-peptide. The eluted complexes were dialyzed, concentrated, and stored at -80°C . For expression and purification of Glutathione S-transferase (GST) tagged XLF protein, *Escherichia coli* BL21 (DE3) cells were transformed with *pGEX6-GST-XLF* plasmid. The GST-XLF protein was induced with isopropyl- β -D-1-thiogalactopyranoside. The cells were harvested and lysed, and cleared lysate was loaded onto a GSTrap HP column (GE Healthcare, Fairfield, Conn). After washing, the GST-XLF protein was eluted by reduced L-glutathione (Wako Chemicals, Richmond, Va). The eluted proteins were dialyzed, concentrated, and stored at -80°C .

In vitro DSB ligation assay

A 934-bp Cy5-labeled double-strand DNA fragment was PCR amplified from pEGFP-C3 vector by using a set of primers (forward: 5'-Cy5-GTTTC GCCACCTCTGACTTGAGCG-3'; reverse: 5'-GAACCTTCAGGGT-CAGCTTGCCGTAG-3'). The DNA fragment was digested with *NcoI* (TaKaRa). A 545-bp fragment (Cy5 labeled at the 5' blunt end and a 3' overhang end) and a 255-bp fragment (with 4-bp overhangs at each end)

were purified. The complexes were incubated for 30 minutes at 37°C in a 30- μL reaction mixture (66 mmol/L Tris-HCl [pH 7.5], 5 mmol/L MgCl_2 , 1 mmol/L dithiothreitol, and 1 mmol/L ATP) with 50 ng of DNA substrates (the Cy5-labeled 545-bp fragment: the unlabeled 255-bp fragment = 2:3). Reaction mixtures were preincubated for 10 minutes on ice with GST-XLF, and then the LIG4-XRCC4 complex was added at 37°C for 15 minutes. The reactions were terminated by adding 1.5 μL of 10% SDS, followed by purification with the QIAquick PCR Purification Kit (Qiagen). Thirty microliters of the eluted DNA was mixed with $10\times$ loading buffer, incubated at 65°C for 5 minutes, and cooled on ice rapidly. Fifteen microliters of the DNA was run on 1% agarose gel and analyzed by using the Typhoon imager (GE Healthcare).

Analysis of protein stability

HEK293FT cells were transiently transfected with XRCC4 or its mutant plasmids. Cells were treated with cycloheximide (100 $\mu\text{g/mL}$) combined with MG132 (20 $\mu\text{mol/L}$), E64D (20 $\mu\text{mol/L}$), epoxomicin (1 $\mu\text{mol/L}$), or 3-MA (10 mmol/L). Cells were harvested at the indicated time points. Whole-cell lysate was analyzed by means of Western blotting with anti-V5 antibody.

Immunoprecipitation

Immunoprecipitation of ectopically expressed WT or mutant XRCC4 proteins in cell from CSL16NG was performed on cell extracts by using anti-V5 agarose (MBL). Immunoprecipitates were washed 5 times with lysis buffer containing 0.15 or 1 mol/L NaCl and then eluted by using V5-peptide (2 mmol/L, MBL). The eluted proteins were separated on 5% to 20% gradient SDS-PAGE gels, blotted onto polyvinylidene difluoride membranes, and analyzed by using Western blotting.

Confocal fluorescence microscopy

Cells were washed once with PBS (Wako) and fixed with Fix buffer (300 mmol/L sucrose, 2% [vol/vol] formaldehyde, and 0.2% [vol/vol] Triton X-100 in PBS). After a brief wash in PBS, cells were blocked with 10% FBS in PBS for 30 minutes and then incubated for 1 hour with the anti-V5-tag, anti- γH2AX , anti-LIG4, or anti-XRCC4 antibodies at appropriate dilutions. The cells were then washed 3 times with PBS plus 0.05% Tween-20 and incubated with Alexa Fluor 488-conjugated secondary antibody at a dilution of 1:500 for 1 hour. The cells were incubated with 4'-6-diamidino-2-phenylindole dihydrochloride (10 ng/mL) for 15 minutes to visualize nuclei. Cells were then washed, mounted, and examined on an LSM 700 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were taken with the $40\times$ oil immersion objective lens under identical imaging settings.

Survival analysis

Fibroblasts (passage number: 5~9) were irradiated by using a ^{137}Cs γ -ray source at a dose rate of 0.09 Gy s^{-1} . Radiosensitivity was assessed by using colony survival analysis. Cells were trypsinized, irradiated, and plated onto feeder cells prepared 24 hours earlier and left to form colonies for 3 weeks. For XRCC4 complementation experiments, normal (48BR), CSL16NG, and CSL16NG-derived fibroblasts expressing WT XRCC4 were seeded in 96-well plates (BD Falcon). After γ -ray irradiation, cells are incubated for a further 24 hours. Sensitivity to ionizing radiation (IR) was measured by means of recovery of DNA synthesis analysis, as described in the Methods section in this article's Online Repository (recovery of RNA synthesis, unscheduled DNA synthesis, and recovery of DNA synthesis assays; passage number of fibroblasts used: 16~20).

RESULTS

Patient CSL16NG has compound heterozygous mutations in XRCC4

Patient CSL16NG, the first daughter of nonconsanguineous white parents, had significant intrauterine growth failure and microcephaly at birth. She showed slow growth, delayed motor

development, and dysmorphic features. Her stature, developmental delay, deep-set eyes, and other features resembled those of patients with CS. However, she had no history of photosensitivity and relatively slow progression of symptoms. She had progressive ataxia. At 19 years, she was given a diagnosis of low-grade thalamic glioma, which was not removed. She has hyperopia, diabetes mellitus, hypothyroidism, moderate hearing loss, and slurred speech, although her understanding remains good. Her neurology has been described previously (patient 3 in Neilan et al²⁸). She has no significant infectious history and no obvious signs of abnormal immune response. Detailed routine blood immunologic peripheral blood tests did not reveal any abnormality. Blood cell counts, including peripheral blood lymphocyte subtype numbers, and immunoglobulin IgM, IgG, and IgA levels were normal. Autoantibodies were negative, and specific vaccine antigen antibody responses were present (see Table E2 in this article's Online Repository at www.jacionline.org). She is currently 23 years old (weight, 41.4 kg [$<<3$ rd percentile]; head circumference, 42.5 cm [$<<3$ rd percentile]; Fig 1, A).

Because a diagnosis of CS was considered, cellular tests for abnormal responses to UV light were carried out on a fibroblast culture established from a skin biopsy specimen, but no abnormality typical of cells from patients with CS was observed (see Fig E2, A-C, in this article's Online Repository at www.jacionline.org). Heterozygous mutations in 2 CS-related genes (*ERCC6/CSB* and *ERCC5/XPG*) were identified, but both alleles were inherited from the asymptomatic father. Both proteins were expressed at normal levels (see Fig E2, D and E). Whole-exome sequencing was carried out, and of the 5 potentially pathogenic candidate genes identified (details are described in the Methods section and Table E1), *XRCC4* was the strongest. One mutation (c.C673T, p.R225*) introduced a premature stop codon; the second mutation (c.G760del, p.D254fs*68) was a frameshift (Fig E3, A, confirmed by means of Sanger gDNA sequencing; see Fig E3, A, in this article's Online Repository at www.jacionline.org), which predicted the expression of a protein with an aberrant C-terminal 68 amino acids, resulting in a 322-amino-acid protein (a normal full-length protein has 334 amino acids).

Allele-specific quantitative RT-PCR using primers that selectively amplify the WT or mutant *XRCC4* cDNAs (see Fig E3, B, and Table E3 in this article's Online Repository at www.jacionline.org) revealed extremely low expression of the p.R225* allele, which is consistent with strong nonsense-mediated mRNA decay (NMD) caused by the premature stop codon (Fig E3, C, middle panel, and Fig E3, D, left panel). The other allele encompassing the p.D254fs*68 frameshift mutation (designated as *XRCC4-Fs* hereafter) was expressed at approximately 30% of the total *XRCC4* expression level in normal 48BR cells (50% would be expected if fully expressed from 1 allele). The small decrease in the frameshift transcript level could reflect some NMD, although we note that the newly generated premature termination codon is located in the last exon, which does not normally trigger NMD. The mutational change could additionally affect the splicing efficiency or accuracy, which might also cause NMD (see Fig E3, D, middle panel, and Fig E3, C, left panel). No detectable *XRCC4* was observed by means of immunoblotting of CSL16NG's cells with a mouse anti-*XRCC4* antibody with an epitope located close to the N-terminus of *XRCC4* (Fig 1, B). We could detect *XRCC4* from a control fibroblast diluted 10-fold, arguing that there is less than 10% of the normal level of *XRCC4* in the patient's cells.

XRCC4 is reported to costabilize *LIG4*.^{5,29,30} Consistently, *LIG4* levels were significantly reduced in fibroblasts from patients CSL16NG, as assessed by means of immunoblotting (Fig 1, B and C). The *LIG4* levels appeared to vary depending on conditions and antibodies (see Fig 1, B, second and third panels). When compared with the *LIG4*-null cell line N114P2 (*LIG4*^{-/-}), a *LIG4* band was clearly visible (Fig 1, C), and from the results of many experiments, we estimate a residual level of between 5% and 15% of normal. We also analyzed *XRCC4* expression by means of immunofluorescence, which revealed a low signal in CSL16NG's cells detectable in both the cytoplasm and nucleus (Fig 1, D).

CSL16NG's cells were radiosensitive and DSB repair defective

Primary fibroblasts from CSL16NG showed marked radiosensitivity relative to control cells (Fig 2, A, and see Fig E4 in this article's Online Repository at www.jacionline.org) and reduced recovery of replication after radiation exposure (Fig 2, B). Furthermore, CSL16NG's cells showed diminished DSB repair capacity, as assessed by enumerating γ H2AX foci (Fig 2, C, compare red and white bars). To confirm that these defects are attributable to the mutation in *XRCC4*, we analyzed the ability of WT *XRCC4* to correct the defect in CSL16NG's cells. When lentivirus-expressing *XRCC4*-Wt cDNA was used to infect these cells, both the ability to synthesize DNA after irradiation (Fig 2, B) and the repair of DSBs (Fig 2, C, green bars) were restored to normal levels.

CSL16NG's cells were defective in a plasmid DSB rejoining assay

We next examined the fidelity of repair in CSL16NG's cells by using an NHEJ end-joining assay that involved transfection of both the patient's cells and control cells with linearized blunt-end plasmids encompassing a 6-bp identical repeat at both DNA ends.²³ Whereas the junctions from control cells were preferentially repaired by means of direct end-joining, this type of repair was nearly absent in CSL16NG's cells (2% vs 72% in control cells, Table I).^{16,24,31,32} Instead, repair of the patient's cells was mediated by the use of 6-bp microhomology (78% vs 8% in control cells) at the junctions, which is consistent with the loss of efficient NHEJ in CSL16NG's cells. A similar skewed repair pattern has previously been observed in XLF-deficient cells.²⁴ These findings demonstrate a marked effect of the mutation on the fidelity of DSB repair.

CSL16NG's cells carried out V(D)J recombination with enhanced fidelity compared with control cells

Defects in NHEJ are usually associated with immunodeficiency. Surprisingly, CSL16NG has no history of chronic infections, and clinical analysis verified a normal immune response (see above). To assess the capacity of CSL16NG's cells to carry out V(D)J recombination, we cotransfected these cells with RAG1/2-expressing plasmids and a V(D)J substrate and assessed the fidelity of recombination.²³ Strikingly, in contrast to the NHEJ assay, most coding junctions were accurately rejoined with little use of microhomology or deletion formation (Table II). This novel V(D)J

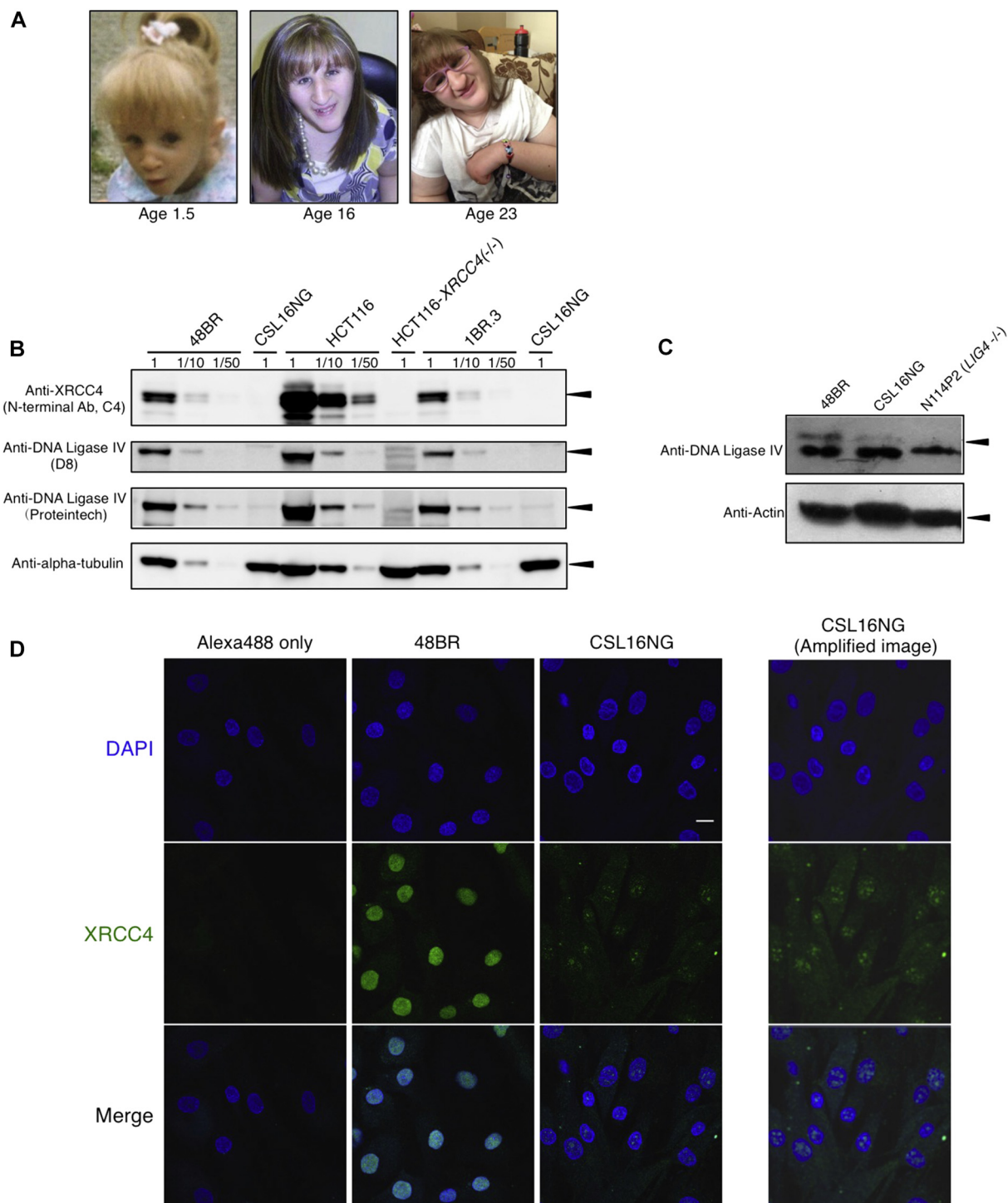


FIG 1. Identification of mutations in the *XRCC4* gene in CSL16NG. **A**, Clinical pictures of CSL16NG. **B**, Immunoblotting of XRCC4 and LIG4 proteins in indicated cells. Dilutions indicate that the amount of XRCC4 in the patient's cells is less than 2% (detection limit) of normal cells. **C**, Immunoblots showing expression of LIG4 proteins in 48BR, CSL16NG, and N114P2 (LIG4^{-/-}) cell lines. **D**, Immunofluorescent staining of endogenous XRCC4 protein using XRCC4 antibody. DAPI, 4'-6-Diamidino-2-phenylindole dihydrochloride.

recombination phenotype contrasts with the pattern obtained with cell lines derived from most patients with LIG4 syndrome and XLF deficiency, where coding junctions are characterized by increased deletions and microhomology use.^{16,18,23,33}

CSL16NG's cells showed an altered repair pattern during CSR

To assess CSR, we examined PBMCs from CSL16NG. The patient was aged 22 years when tested, and thus we compared her CSR junctions with our previously published 154 S μ -S α

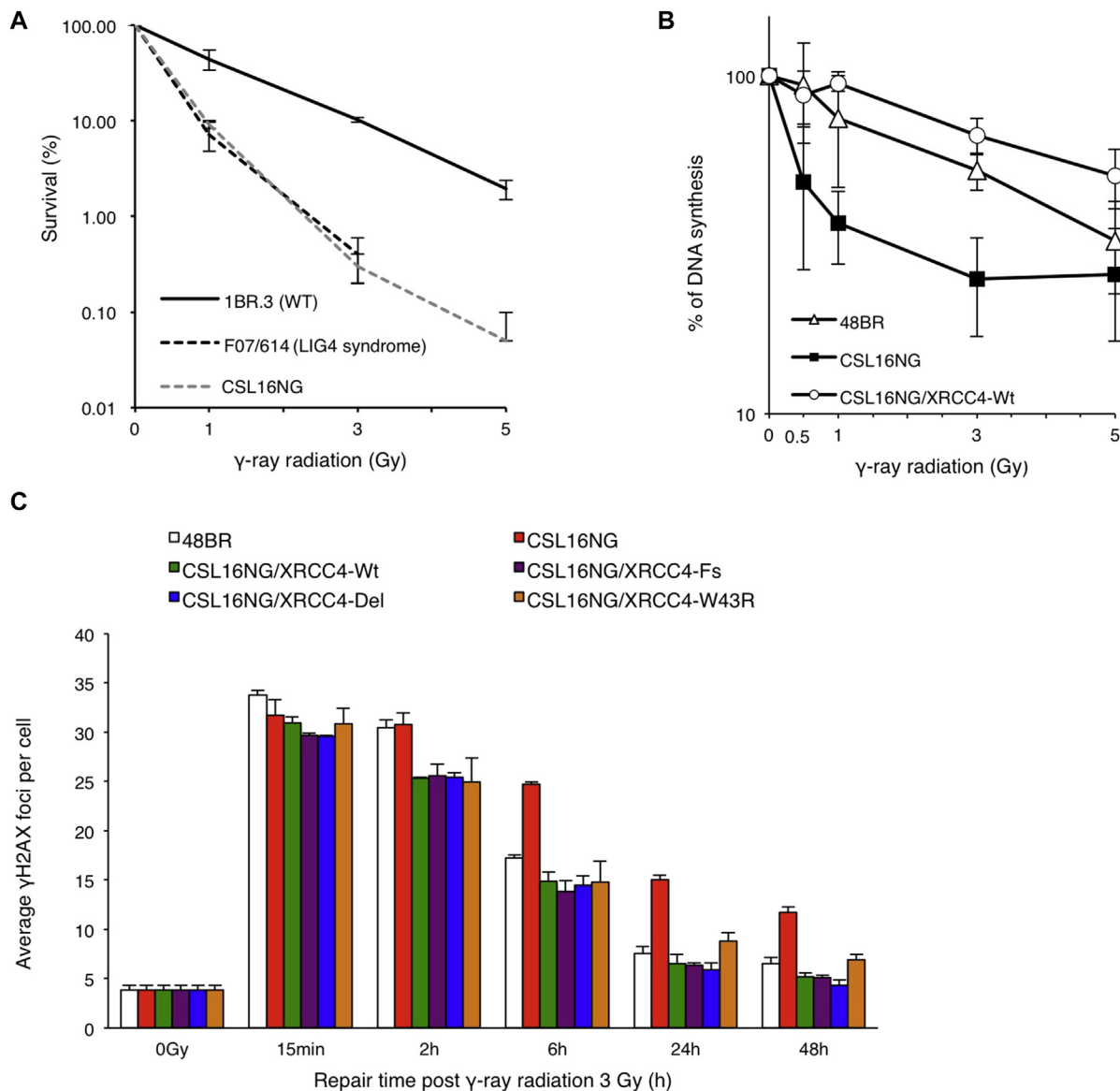


FIG 2. CSL16NG's defects in DNA DSB repair. **A**, The colony-forming ability of CSL16NG's cells after γ -irradiation was compared with that of normal (1BR.3) and *LIG4*-defective F07/614 cells. **B**, Rescue of the inability of CSL16NG's cells to arrest DNA synthesis after IR by using WT XRCC4 overexpression. **C**, Complementation of the DSB repair defect in CSL16NG's cells by expression of WT or mutant XRCC4 proteins. Error bars = SDs of triplicate experiments.

junctions from healthy adults (Table III and see Fig E5 in this article's Online Repository at www.jacionline.org).³⁴ Amplification of $S\mu$ - $S\alpha$ junctions from *in vivo*-switched B lymphocytes from the patient revealed a reduced frequency of junctions repaired by using direct end-joining, although not to a significant degree (5% vs 18% in control cells; $P = .15$, χ^2 test), and a markedly increased frequency of repair by unusually long microhomologies (≥ 10 bp; 36% vs 3% in control cells). The altered pattern was similar to but less prominent than that previously observed in XLF- and LIG4-deficient patients.^{24,34} Thus although the patient has a normal serum IgA level, the altered pattern of the CSR junctions still clearly suggests a defect in the recombination process, which relies on classical NHEJ machinery.

Effect of the XRCC4-Fs mutation on XRCC4 function

We next examined the effect of XRCC4-Fs on XRCC4 function and protein expression. We also examined 2 other forms of XRCC4 protein (see Fig E6, A, in this article's Online Repository at www.jacionline.org): XRCC4-Del has a stop codon at the position of the frameshift mutation in CSL16NG and thus lacks the C-terminal aberrant polypeptide. XRCC4-W43R contains an amino acid substitution p.W43R previously identified in a patient with primordial dwarfism.³⁵ Although Trp43 is conserved, there is no current evidence for causality. After lentivirus infection, as described above, we found that all forms of the protein were expressed (see Fig E6, B). Strikingly, normal DSB repair activity was restored not only by expression of WT XRCC4 but also by XRCC4-Fs. Furthermore, XRCC4-Del also complemented

TABLE I. Increased microhomology-dependent repair in CSL16NG's cells in the plasmid-based NHEJ assay*

Cell line	Direct end-joining	6-bp MH	Deletion + MH	Deletion only	Total no. of junctions
CSL16NG	1 (2%)#↓	47 (78%)#↑	3 (5%)	9 (15%)	60
XLF ^{-/-} †	3 (4%)#↓	55 (81%)#↑	10 (15%)	0 (0%)¶↓	68
411BR LIG4 deficient‡	4 (22%)#↓	12 (67%)#↑	0 (0%)	2 (11%)	18
Control§	105 (72%)	11 (8%)	14 (10%)	16 (11%)	146

MH, Microhomology.

*Statistical calculations were performed by using the χ^2 test, and significant changes are indicated in boldface.†Previously published results from the XLF-deficient cell line.²⁴‡411BR cells, as described in O'Driscoll et al¹⁶ and Frank et al.³¹§Newly generated results merged with previously published results from the control cell line.^{24,32}¶ $P < .01$.# $P < .001$.**TABLE II.** Increased fidelity of end-joining at coding junctions in CSL16NG in plasmid-based VDJ-assay*

Cell line	Accurate end-joining	4-bp MH	Deletion + MH	Deletion only	Total no. of junctions
CSL16NG	18 (90%)§↑	0 (0%)†↓	2 (10%)‡↓	0 (0%)†↓	20
XLF ^{-/-}	5 (12%)	4 (10%)	32 (78%)§↑	0 (0%)§↓	41
Control	13 (10%)	25 (20%)	57 (46%)	30 (24%)	125

MH, Microhomology.

*Statistical calculations are performed by using the χ^2 test, and significant changes are indicated in boldface.† $P < .05$.‡ $P < .01$.§ $P < .001$.**TABLE III.** Characterization of S μ -S α junctions*

Patients	Perfectly matched short homology						Total no. of S fragments
	0 bp		1-3 bp	4-6 bp	7-9 bp	≥10 bp	
	Direct end-joining	Small insertions					
CSL16NG (n = 1)	1 (5%)	2 (9%)	4 (18%)	2 (9%)	5 (23%) ‡↑	8 (36%) ↑	22
LIG4-deficient patients† (n = 2)	0 (0%) ‡↓	1 (3%) §↓	7 (23%)	4 (13%)	4 (13%)	14 (47%) ↑	30
Control cells‡ (n = 17)	28 (18%)	39 (25%)	56 (36%)	15 (10%)	11 (7%)	5 (3%)	154

*Statistical analysis was performed by using the χ^2 test, and significant differences are indicated in boldface.†Previously published junctions from LIG4-deficient patients and control subjects.³⁴‡ $P < .05$.§ $P < .01$.|| $P < .001$.

CSL16NG's DSB repair defect, whereas XRCC4-W43R showed little complementation (Fig 2, C, purple, blue, and orange bars). Lentiviral expression results in XRCC4 overexpression. To examine DSB repair under physiologic expression levels, we also used human telomerase reverse transcriptase-immortalized cell from CSL16NG in a plasmid-based complementation assay (see Fig E7 in this article's Online Repository at www.jacionline.org) and measured DSBs as 53BP1 foci in cells containing the plasmid. Full complementation was found by using WT XRCC4 protein, and substantial, although not full, correction was found by using XRCC4-Fs (see Fig E7, B). Untransfected cells in the same population showed the expected DSB repair defect.

These findings suggest that the mutant protein can partially function in DSB repair. This function likely contributes to the residual DSB repair capacity observed in CSL16NG's cells. We also conclude that the W43R mutation impairs XRCC4 function.

Biochemical characterization of the mutant XRCC4 proteins

To further examine the functional capacity of XRCC4-Fs, we purified recombinant XRCC4-LIG4 complexes and examined their activity *in vitro*. Flag-tagged XRCC4-Wt, XRCC4-Fs, and

XRCC4-Del proteins were coexpressed with WT His-tagged LIG4 in HEK293FT cells and copurified by means of affinity chromatography (see Fig E8, A, in this article's Online Repository at www.jacionline.org; GST-XLF was purified from *E coli* in Fig E8, B). XRCC4-Fs and XRCC4-Del formed complexes with LIG4, demonstrating that the XRCC4 C-terminus is dispensable for complex formation.

In vitro ligation assays were performed with a fluorescently labeled double-strand DNA with a 3' overhang on one side and an additional smaller double-strand DNA substrate with the same 3' cohesive overhang on both sides (see Fig E8, C). The ability of the XRCC4-Fs and XRCC4-Del mutants to support LIG4-mediated ligation was indistinguishable from that of XRCC4-Wt. Furthermore, the activity was efficiently stimulated by XLF. We conclude that the effect of these alterations on XRCC4/LIG4 enzyme activity is relatively minor. This finding explains the substantial complementation when mutant proteins are exogenously expressed.

XRCC4-Fs is degraded by the proteasome

Given the low levels of XRCC4-Fs observed in CSL16NG's cells and its presence in cytoplasm, we examined whether it is

subject to proteolytic degradation. To assess this, we first transiently expressed WT and mutant XRCC4 in HEK293FT cells in the presence or absence of the protein synthesis inhibitor cycloheximide. In the presence of cycloheximide, XRCC4-Wt protein was stable up to 12 hours, whereas XRCC4-Fs was significantly degraded (see Fig E9, A, in this article's Online Repository at www.jacionline.org). Remarkably, the XRCC4-Del protein was as stable as XRCC4-W43R. Degradation of XRCC4-Fs was inhibited by addition of the proteasome inhibitors MG-132 and epoxomicin (see Fig E9, B) but unaffected by the cysteine protease inhibitor E64D, which inhibits calpain and lysosomal proteases, or 3-MA, an autophagy inhibitor (see Fig E9, B). Consistent with these observations, the level of endogenous XRCC4 in CSL16NG's cells was significantly enhanced by incubation in MG132 (see Fig E10 in this article's Online Repository at www.jacionline.org). These findings strongly suggest that XRCC4-Fs is subjected to proteasome-mediated degradation, which provides an explanation for the very low (indeed undetectable) level of the mutant XRCC4 protein and the low level of LIG4 in the patient's cells. In view of these findings, we predicted that the enhanced expression of exogenous WT or mutant XRCC4 in CSL16NG's cells would lead to increased LIG4 levels. Indeed, overexpression of WT and XRCC4 mutant proteins in CSL16NG resulted in recovery of LIG4 protein levels (see Fig E9, C, compare lanes 3-6 with lane 2), which is consistent with the ability of XRCC4-Fs to complement the repair defect found in CSL16NG.

Taking the complementation analyses and biochemical data together, we conclude that the XRCC4-Fs mutant protein can efficiently complex with LIG4 and promote DSB rejoining and that a major effect of the change is the greatly reduced XRCC4-Fs stability caused by proteasomal degradation.

Mutations in CSL16NG confer a separation of impact phenotype

Despite the DSB repair defect observed in CSL16NG's cells and marked developmental features, the patient had a normal immune response, and the patient's cells showed enhanced accuracy in an *in vitro* assay for V(D)J recombination, which is in marked contrast to other NHEJ-deficient patients. A trivial explanation might be that there is sufficient residual DSB repair activity to support V(D)J recombination but not the efficient repair of radiation-induced DSBs. To gain further insight, we carried out a comparative analysis of the efficiency of DSB repair of CSL16NG's cells and a range of patient-derived fibroblasts with deficiencies in other NHEJ proteins (XLF or LIG4; Fig 3, A). Because LIG4 is essential, patients with LIG4 syndrome have hypomorphic mutations and hence a range of clinical severities, which generally correlate with the magnitude of the repair defect.³⁶ Importantly, most of the cells from patients with LIG4 syndrome that we examined (and in total 7/8 lines previously examined by us) repaired most DSBs induced by 3 Gy of IR by 72 hours (Fig 3, A, and data not shown). (495GOS had more marked sensitivity. However, this line had normal LIG4 protein expression, raising the possibility that the mutational change could exert a dominant negative phenotype.) Except for patient 180BR,^{37,38} all showed some degree of immunodeficiency. Of relevance here, the *in vitro* ligation activities of cell lines 411BR and 2303 have been previously estimated to be less than 1%, yet both lines rejoin the DSBs induced by 3 Gy within 72

hours.^{36,39} 411BR has previously been shown to express normal levels of mutant LIG4,^{36,39} but significantly, 2303 cells expressed lower levels of LIG4 compared with CSL16NG's cells (Fig 3, B and C). Collectively, this analysis of cell lines from patients with LIG4 syndrome strongly suggests that only a low level of residual LIG4 activity ($\leq 1\%$) suffices to repair all DSBs induced by 3 Gy of IR, although with slow kinetics, a conclusion consistent with previous findings.⁴⁰ In marked contrast, CSL16NG's fibroblasts, despite harboring 5% to 15% residual LIG4 levels had a severe DSB repair defect. The magnitude of the DSB repair defect was similar to but more severe than that shown by XLF-defective cells. The repair defect was evident after exposure to a low dose of radiation (0.5 Gy), and after 3 Gy of IR, 25% of the DSBs remained unrepaired, even 8 days after exposure (see Fig E11, A and B, in this article's Online Repository at www.jacionline.org). Indeed, this represents possibly the most severely DSB repair defective human cell line that we have encountered. In rodent cells defects in NHEJ proteins, including XLF, can lead to DSB repair by alternative NHEJ.⁴¹ However, the residual DSB repair in CSL16NG's cells is not inhibited by the poly ADP ribose polymerase inhibitor KU58958 (see Fig E11, C) and displays an additive repair defect after small interfering RNA depletion of LIG4 (see Fig E11, D) or addition of the DNA-PK inhibitor NU7441 (see Fig E11, C), suggesting that it does not represent alternative NHEJ but residual canonical NHEJ activity. This is consistent with current evidence that alternative NHEJ does not function in human cells unless Ku is absent.^{42,43} We note also that CSL16NG's cells expressed close to normal levels of XLF, as did the cells from patients with LIG4 syndrome that we examined (Fig 3, B).

These findings show that CSL16NG has a very severe defect in repair of DSB, and importantly, they suggest that the normal immune response of the patient cannot be attributed to a mild defect in DSB repair, providing evidence for a separation of function phenotype.

DISCUSSION

We describe a patient with mutational changes in the NHEJ protein XRCC4. The patient described here has unexpected clinical features for an NHEJ deficiency. Our detailed cellular analysis provides evidence for a separation of impact phenotype, namely severely defective DSB repair but normal immune responses, and reveals a clinical and cellular manifestation for mutations in XRCC4 that is unexpected and distinct from those conferred by diminished LIG4 enzyme activity.

In patient CSL16NG the major expressed allele encodes a protein with 68 aberrant C-terminal amino acids p.(D254fs*68). The mutant protein can interact with LIG4 and is largely functional for DSB repair *in vitro* and *in vivo*. The major effect of the mutational change is to promote proteasome-mediated degradation of the mutant protein, so that CSL16NG's cells have very low XRCC4 protein levels.

There are 2 remarkable findings from our results, with important implications. First, the defect in DSB repair is significantly greater than that of cells with lower LIG4 levels, and second, despite this defect, V(D)J recombination is proficient and the patient does not display any gross clinical or laboratory immunodeficiency to date. We will consider these findings in turn.

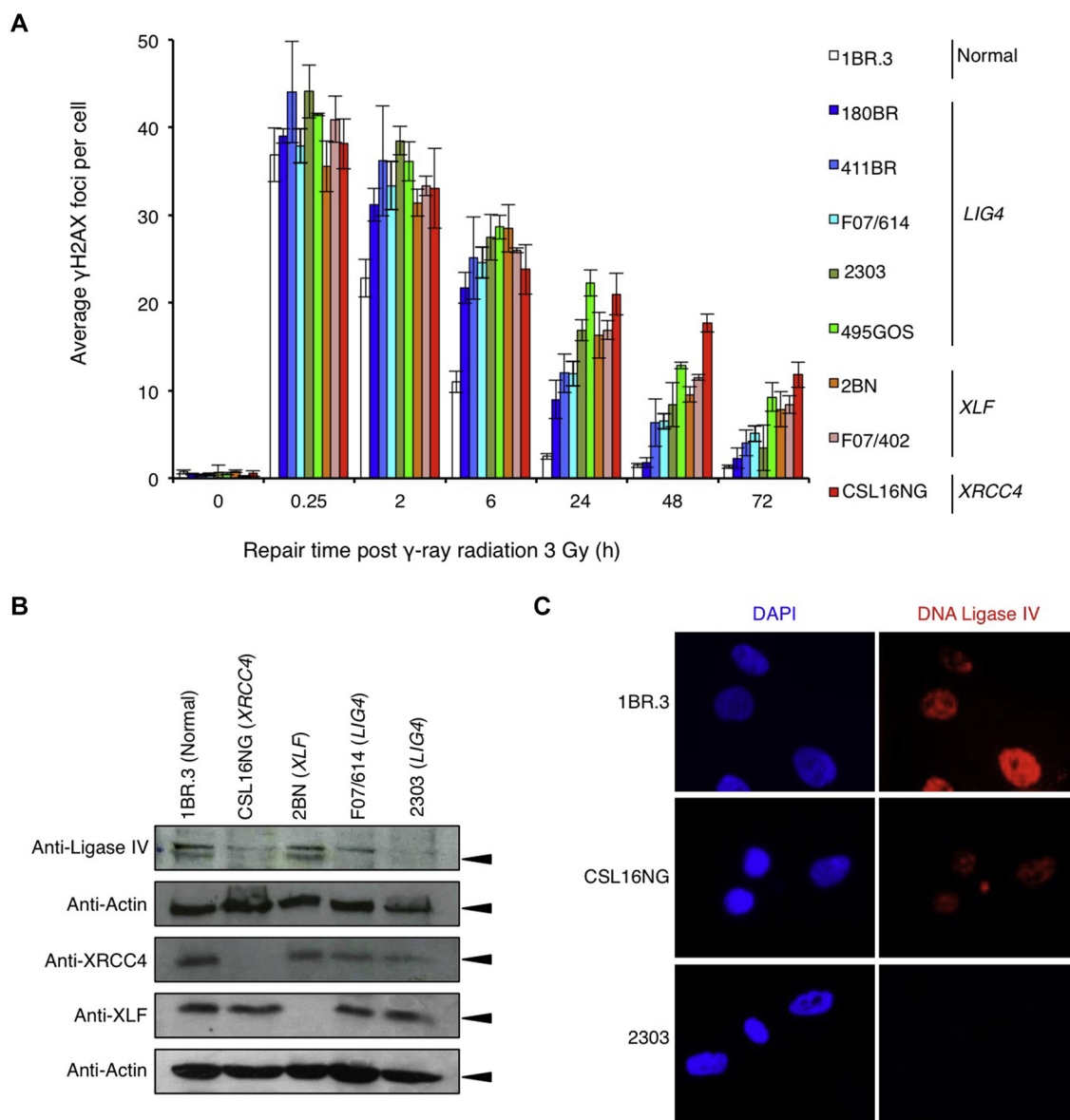


FIG 3. CSL16NG's cells show an exceptionally marked DSB repair defect. **A**, DSB repair kinetics after IR were determined in normal and various DSB repair-deficient cell lines. Passage number of all fibroblasts used: 5–9. Error bars = SDs of triplicate experiments. **B**, Immunoblotting of XRCC4, LIG4, and XLF proteins in normal cells (1BR.3), the patient's cells (CSL16NG), cells from a patient with XLF deficiency (2BN), and cells from a patient with LIG4 syndrome (F07/614 and 2303) cells. **C**, Diminished LIG4 expression in CSL16NG's cells was confirmed by means of immunofluorescent staining. DAPI, 4'-6-Diamidino-2-phenylindole dihydrochloride.

DSB repair defect

Despite low XRCC4 levels, there is approximately 5% to 15% residual LIG4 localized in the nucleus. This residual activity, by comparison with cells from patients with LIG4 syndrome, is estimated to be sufficient to affect slow DSB rejoining after 3 Gy of IR, yet CSL16NG's cells have a dramatic DSB repair defect. Therefore these findings strongly suggest that there must be a role for XRCC4 in DSB rejoining that is distinct from its accepted function in stabilizing LIG4.

Recent studies have shown that XLF forms filaments with XRCC4, and current models propose that these function in DNA end-stabilization during DSB repair.^{8,10-12} This structural

role for XRCC4 in forming filaments necessitates multiple XRCC4 molecules at each DNA end and is unlikely to be fulfilled by a low level of residual XRCC4 protein. The similar DSB repair defect between XLF-deficient cells and CSL16NG's cells supports an overlapping role in DSB repair. Thus we propose that the marked DSB repair defect in CSL16NG's cells is a consequence of insufficient XRCC4 to form filaments with XLF. In contrast, low levels of residual LIG4, of which only 1 to 2 molecules are likely to be required per DSB, is sufficient to allow complete DSB repair, although with slow kinetics (see Fig E11, B). If this hypothesis is correct, our data provide the first evidence suggesting that

XRCC4's role in XLF filament formation can be uncoupled from its more direct role in ligation.

DSB repair defect and normal immune response

Despite the marked DSB repair defect in CSL16NG's cells and an altered repair pattern during CSR, the patient did not display any immunodeficiency, and in a cell-based assay V(D)J recombination took place with enhanced rather than reduced accuracy. Most cells from patients with LIG4 syndrome, including line 2303, displayed CID or SCID, yet our comparative analysis of DSB repair showed that most cells from patients with LIG4 syndrome had more efficient DSB repair than CSL16NG's cells.¹⁶ Thus the normal immune response of CSL16NG cannot be attributed solely to a mild DSB repair defect, suggesting a separation of impact phenotype.

This raises the intriguing possibility that XRCC4's role in complex with XLF in end-stabilization might be dispensable for V(D)J recombination. However, XLF-deficient patients display immunodeficiency, demonstrating a role for XLF in V(D)J recombination in human subjects.¹⁸ There appear to be distinctions between the requirement for synapsis/end-stabilization during V(D)J recombination versus the repair of genotoxic DSBs. Indeed, XLF is dispensable for V(D)J recombination in mice, whereas ATM, DNA-PK, and 53BP1 have been proposed to have redundant roles, yet XLF-deficient patients frequently display pronounced immunodeficiency.^{44,45} We note that the phenotype of CSL16NG's cells overlaps with that of XLF-deficient mice (ie, a marked DSB repair defect but proficient V(D)J recombination).¹⁵ Additionally, in mice RAG2 has been proposed to have a role in influencing rejoining during V(D)J recombination.⁴⁶ We note also that a recent article identified PAXX as a new XRCC4 superfamily member that functions with XRCC4 and XLF in NHEJ.⁴⁷ It is possible that there could be redundancy between PAXX and XRCC4 during V(D)J recombination but not during repair of genotoxic DSBs. Further work is required to gain insight into the mechanism underlying these intriguing findings.

An additional novel phenotype of CSL16NG's cells is that all the junctions formed in the V(D)J recombination assay involved high-fidelity joining of the coding ends. This phenotype contrasts strongly with the lack of direct rejoining of DNA ends in the NHEJ plasmid assay and CSR junction analysis and further supports a separation of function phenotype. The basis for the difference from control cells in the V(D)J recombination assay, where small deletions/insertions can arise, is unclear. However, the accurate rejoining contrasts to other NHEJ-deficient lines, which are characterized by large deletions and microhomology use. Although the lack of junctional diversity seen in this fibroblast-based assay might predict a diminished repertoire of T and B cells, it is possible that the assay does not fully reflect the *in vivo* process because of differences between fibroblasts and lymphocytes (eg, T cells express terminal deoxynucleotidyl transferase).

Using CSL16NG's fibroblasts and the assays established here, we also examined the effect of W43R, a previously described XRCC4 mutational change observed in a patient with primordial dwarfism.³⁵ We provide evidence that W43R is a pathologic mutational change, supporting the notion that homozygous expression of XRCC4-W43R underlies the primordial dwarfism observed in the patient.

Several recent studies have also described patients with mutations in XRCC4.⁴⁸⁻⁵¹ Strikingly, all patients displayed microcephaly and growth delay but no overt immunodeficiency (although detailed examination of V(D)J recombination ability was not investigated in these patients). This strengthens our proposal that XRCC4 deficiency confers a separation of impact phenotype (impacting on the repair of genotoxic DSBs but not the repair of DSBs induced during V(D)J recombination), demonstrating that this is not a finding specific to our patient. The severe DSB repair defect in CSL16NG's cells also provides a potential explanation for the marked neurological phenotype observed in the patient. An unusual feature for NHEJ deficiency was the development of progressive ataxia in the patient, and it is tempting to speculate that this is a consequence of persisting unrepaired DSBs. Intriguingly, a progressive neurological phenotype involving unsteady gait was recently observed in 2 XRCC4-deficient siblings, who similarly had no detectable residual XRCC4 protein.⁵¹ This supports the notion that progressive ataxia could be a consequence of marked XRCC4 deficiency.

The prediction of our working model is that mutations in XRCC4 that result in low expression of a protein capable of interacting with LIG4 will confer marked neurological deficits without associated immune deficiency. Given the striking resemblance of patient CSL16NG when a young child to patients with CS, XRCC4 should be considered a candidate for potential causal mutations in further patients with similar features, including further CS-like patients with normal transcription-coupled nucleotide excision repair.

In summary, we describe a patient with mutations in XRCC4. The patient had marked neurological abnormalities with progressive ataxia. Our biochemical and cellular analysis provides evidence that the mutational changes in this patient confer a separation of impact phenotype with a severe defect in DSB repair but no marked defect in V(D)J recombination. Recent articles have described additional XRCC4-deficient patients with a normal immune response.^{35,48-51} As a working model, we propose that this could arise from a separation of XRCC4 function in stabilizing LIG4 versus its role in forming filaments with XLF. We propose that XRCC4 is required for all DSB rejoining because LIG4 is unstable in the absence of XRCC4. However, only a low level of protein suffices to provide sufficient LIG4 to effect slow rejoining. In contrast, XRCC4's role in filament formation with XLF is essential for efficient rejoining of genotoxic DSBs but appears to be dispensable during V(D)J recombination in human cells, possibly because of redundancy with other proteins that can promote synapsis.

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Clinical implications: Exome sequencing identified pathogenic mutations of XRCC4 in a patient displaying microcephaly and progressive ataxia. Despite the severe cellular radiosensitivity, the patient maintained normal immune response.

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