

# Functional redundancy between the XLF and DNA-PKcs DNA repair factors in V(D)J recombination and nonhomologous DNA end joining

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**Classical nonhomologous end joining (C-NHEJ) is a major mammalian DNA double-strand break (DSB) repair pathway that is required for assembly of antigen receptor variable region gene segments by V(D)J recombination. Recombination activating gene endonuclease initiates V(D)J recombination by generating DSBs between two V (D)J coding gene segments and flanking recombination signal sequences (RS), with the two coding ends and two RS ends joined by C-NHEJ to form coding joins and signal joins, respectively. During C-NHEJ, recombination activating gene factor generates two coding ends as covalently sealed hairpins and RS ends as blunt 5'-phosphorylated DSBs. Opening and processing of coding end hairpins before joining by C-NHEJ requires the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). However, C-NHEJ of RS ends, which do not require processing, occurs relatively normally in the absence of DNA-PKcs. The XRCC4-like factor (XLF) is a C-NHEJ component that is not required for C-NHEJ of chromosomal signal joins or coding joins because of functional redundancy with ataxia telangiectasia mutated kinase, a protein that also has some functional overlap with DNA-PKcs in this process. Here, we show that XLF has dramatic functional redundancy with DNA-PKcs in the V(D)J SJ joining process, which is nearly abrogated in their combined absence. Moreover, we show that XLF functionally overlaps with DNA-PKcs in normal mouse development, promotion of genomic stability in mouse fibroblasts, and in IgH class switch recombination in mature B cells. Our findings suggest that DNA-PKcs has fundamental roles in C-NHEJ processes beyond end processing that have been masked by functional overlaps with XLF.**

Cernunnos | nej1 | nhej1 | SCID | T-FISH

**D**ouble-strand break (DSB) repair is crucial for maintaining stability of the mammalian genome. There are two major DNA DSB repair pathways in mammalian cells. Homologous recombination repairs DSBs with high fidelity and is active in the S/G2 phases of the cell cycle. Classical nonhomologous end joining (C-NHEJ) rejoins DSBs, often imprecisely, throughout the cell cycle (1, 2). During C-NHEJ, the Ku70/Ku80 heterodimer (Ku) recognizes the DSB and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form a DNA-dependent protein kinase holoenzyme (DNA-PK). DNA-PK activates the Artemis endonuclease, which can contribute to the processing of DSB ends, if necessary, to allow their ligation (3). Ku70/Ku80 also recruits the XRCC4/DNA ligase 4 (Lig4) complex that ligates DSB ends. Ku70, Ku80, XRCC4, and Lig4 are considered to be "core" C-NHEJ factors because they are required to repair all types of DSB ends. However, deficiency for Ku70, Ku80, XRCC4, Lig4, Artemis, or DNA-PKcs abrogates lymphocyte development and leads to severe combined immunodeficiency (SCID) in mice due to their requisite role in coding join (CJ) formation (1).

During the B and T lymphocyte development, exons that encode Ig (Ig) and T-cell receptor variable regions are assembled from V, D, and J gene segments. This V(D)J recombination process is initiated by the RAG1/RAG2 (RAG) endonuclease (4). V, D, and

J coding segments are each flanked by RSs that target RAG. RAG cleaves between the borders of two appropriately paired coding segments and their flanking recombination signal sequences (RSs) to generate two hairpin-sealed coding ends (CEs) and two blunt 5'-phosphorylated RS ends (SEs). The SEs are ligated back together without further processing, whereas the coding ends must be opened and processed before they can be joined. Both CE and SE are joined by C-NHEJ pathway. In this context, Ku and the XRCC4/Lig4 complex are required for both CJ and signal joins (SJ) formation. However, although DNA-PKcs and Artemis are absolutely required for CJ formation due to their role in opening and processing these ends, RS join formation can occur in their absence as these ends do not require further processing (1, 2, 4). IgH class switch recombination (CSR) is a second type of programmed genome rearrangement that occurs in activated mature B cells to allow expression of different classes of antibodies. CSR is initiated by activation-induced cytidine deaminase, the activity of which results in DSBs in large switch (S) regions flanking different sets of IgH constant region exons. These DSBs are joined between two different S regions by C-NHEJ, or in the absence of C-NHEJ, at reduced levels by alternative end-joining pathways (1). DNA-PKcs is required for fully normal CSR (5–9).

DNA-PKcs mutations in mice lead to severe combined immune deficiency due to inability to form V(D)J CJs (10–12). Both point mutations in the kinase domain of DNA-PKcs (SCID mice) and elimination of DNA-PKcs expression by gene-targeted mutation abrogate CJ formation (13–15). However, in the absence of DNA-PKcs, SJ formation occurs at near normal levels in embryonic stem cells and pro-B cells (15, 16), but occurs at reduced levels with decreased fidelity in various nonlymphoid cells (17–19). During V(D)J recombination, DSBs activate ataxia telangiectasia mutated (ATM) kinase (4). ATM belongs to the same family of protein kinases as DNA-PKcs; and, indeed, these two kinases have common substrates (i.e., histone H2AX). ATM is required for fully normal chromosomal CJ formation, but it is dispensable for SJ formation (20). Combined deficiency for ATM and DNA-PKcs in mice leads to embryonic lethality (21, 22). Moreover, ATM and DNA-PKcs have redundant functions in SE joining in pro-B cells (16, 23). When both proteins are inactivated, or when ATM-deficient and DNA-PKcs-deficient pro-B cells are treated with DNA-PKcs and ATM specific inhibitors, respectively, SE joining is greatly impaired (16, 23). ATM and DNA-PKcs also have redundant functions in CSR, as switching to IgG1 and IgG3 was

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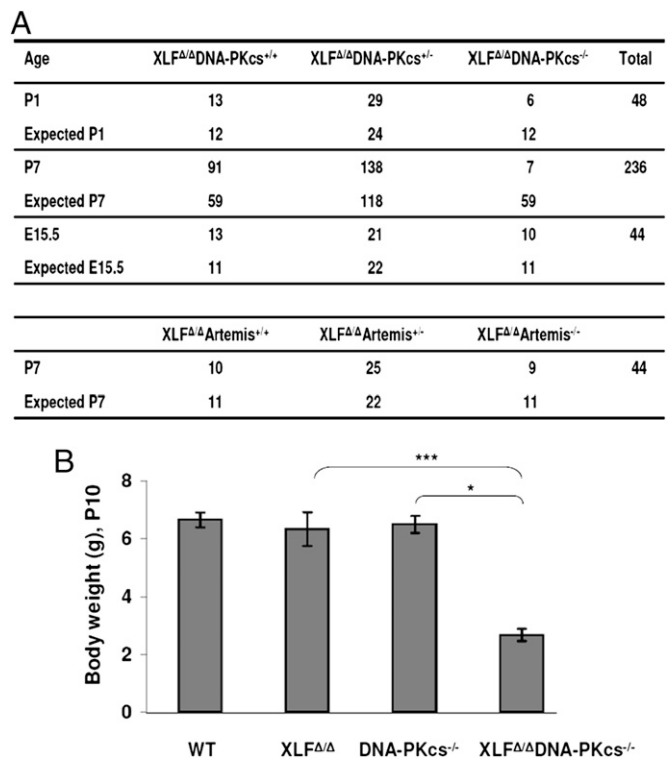
reduced in ATM-deficient B cells when treated with DNA-PKcs inhibitors (8).

The XRCC4-like factor (XLF; also called Cernunnos in humans, or nej1 in yeast) is a C-NHEJ factor that is recruited by Ku (24) and has structural relatedness to and interacts with XRCC4 (25). Human XLF deficiency leads to immunodeficiency and developmental defects (26, 27). Correspondingly, embryonic stem cells and fibroblasts from XLF-deficient mice have ionizing radiation sensitivity, genomic instability, and impaired extrachromosomal V(D)J recombination (28). In addition, CSR is reduced in XLF-deficient mouse B cells (29). However, chromosomal V(D)J recombination is largely normal in developing lymphocytes and pro-B-cell lines from XLF-deficient mice (29, 30) because of XLF functional redundancy with ATM and downstream substrates, including H2AX and 53BP1 in C-NHEJ (31–33). Thus, XLF/ATM and XLF/53BP1 double-deficient mice are smaller than single-deficient littermates, have a SCID phenotype associated with severely deficient V(D)J CE and SE joining, and have a greater reduction in CSR than single-deficient mice (31–33). XLF/H2AX double-deficient pro-B lines also have severely impaired chromosomal V(D)J recombination (31). In addition, unjoined CEs and SEs generated during attempted V(D)J recombination in XLF/H2AX and XLF/53BP1 double-deficient pro-B cells are highly resected, consistent with a role for H2AX and 53BP1 in end protection (34–36). It is notable that ATM deficiency or treatment with ATM inhibitors rescues the end resection, but not the V(D)J joining phenotype of XLF/H2AX or XLF/53BP1 double-deficient pro-B lines (31, 32); this is consistent with ATM having both a role in end joining and in activating the CtIP nuclease for end resection (34). Potential roles for XLF in end protection have been speculated but not tested.

## Results

**Combined Deficiency of DNA-PKcs and XLF Affects Mouse Growth and Viability.** Mice with combined deficiency of DNA-PKcs and XLF (XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup>) were obtained from breeding mice heterozygous for DNA-PKcs and homozygous for an XLF null allele (XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>+/-</sup>). XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup> embryos occurred at approximately expected ratios at E15.5 (Fig. 1A), showing that the double-deficient genotype does not result in early embryonic lethality. However, XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup> mice appeared to be born at sub-Mendelian ratios and few survived past P7 (Fig. 1A) and none of the ones survived past day P10. Live-born XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup> pups were significantly smaller than their littermates (Fig. 1B). Mice with combined deficiency of Artemis and XLF (XLF<sup>Δ/Δ</sup>Artemis<sup>-/-</sup>) were obtained from breeding mice heterozygous for Artemis and homozygous for an XLF null allele (XLF<sup>Δ/Δ</sup>Artemis<sup>+/-</sup>). These mice were born at Mendelian ratios (Fig. 1A) and had no obvious defects beyond expected V(D)J recombination or genomic stability phenotypes associated with one or the other single-deficient genotype (see below). We conclude that XLF and DNA-PKcs have redundant functions in mouse development, reminiscent of those associated with core C-NHEJ deficiencies, and which, correspondingly, are not related to the role of DNA-PKcs in activating Artemis.

**DNA-PKcs kKinase Activity Is Required for V(D)J SJ Formation in XLF-Deficient Pro-B Lines.** Single deficiency for XLF or DNA-PKcs does not lead to major defects in SE joining during chromosomal V(D)J recombination in pro-B-cell lines (16, 31, 32) (Fig. 2A–C). However, double deficiency for XLF and ATM markedly impaired SE joining (31). As DNA-PKcs and ATM belong to the same family of protein kinases, we tested whether XLF has functional redundancy with DNA-PKcs. For this, we generated WT, XLF<sup>Δ/Δ</sup>, DNA-PKcs<sup>-/-</sup>, or XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup> v-Abl kinase transformed (v-Abl) pro-B cells harboring a *bcl-2* transgene. We treated the cells with the v-Abl kinase inhibitor STI571 to arrest the cells in the G1 cell-cycle phase leading to induction of RAG expression and V(D)J recombination. In these experiments, the *bcl-2* transgene allows cells to survive following STI571 treatment (20). Then, we assayed SE joining within a chromosomally integrated V(D)J recombination substrate (pMX-DEL-SJ) via a Southern blotting with



**Fig. 1.** Combined deficiency of DNA-PKcs and XLF significantly affects mouse growth and survival. (A) The number of live-born mice genotyped at day 1 (P1) or day 7 (P7), and number of mouse embryos at day E15.5, from crosses between XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>+/-</sup> mice are indicated (Upper). (Lower) The number of live-born mice (P7) from crosses between XLF<sup>Δ/Δ</sup>Artemis<sup>+/-</sup> mice are indicated. (B) The body weight in grams of the indicated 10-d-old mice (P10) is plotted. The numbers represent the average and SD from at least 3 mice of each genotype. \**P* = 0.01 and \*\*\**P* = 0.0003.

which we can observe bands corresponding to the unrearranged cassette (UR), SEs, and SJs. (ref. 20; Fig. 2A).

Pro-B-cell lines from three different XLF<sup>Δ/Δ</sup>DNA-PKcs<sup>-/-</sup> mice were tested, and all showed the same dramatic phenotype, compared with WT or single mutant lines, which was an accumulation of unjoined SEs consistent with nearly complete abrogation of SE joining (Fig. 2B). To test if this functional redundancy of DNA-PKcs involved its kinase activity, we treated both WT and XLF<sup>Δ/Δ</sup> v-Abl pro-B cells with DNA-PKcs-specific inhibitor Nu-7441. Upon STI571 treatment and RAG induction, the XLF<sup>Δ/Δ</sup> cells failed to join SEs, whereas vehicle-treated XLF<sup>Δ/Δ</sup> cells completed V(D)J recombination as efficiently as WT lines (Fig. 2C). Thus, DNA-PKcs kinase activity is required for efficient SE joining in XLF<sup>Δ/Δ</sup> cells. It is notable that the pro-B lines that were double deficient for XLF and Artemis (XLF<sup>Δ/Δ</sup>Artemis<sup>-/-</sup>) efficiently joined the SEs with very low levels of unjoined SEs consistent with the low levels observed in XLF-deficient cells alone (Fig. 2D; refs. 31, 32). Thus, the redundant functions of DNA-PKcs with XLF in SE joining rely on functions of the DNA-PKcs kinase separate from activation of Artemis.

**DNA-PKcs and XLF Have Redundant Functions During CSR.** We found that XLF and DNA-PKcs have redundant functions in SE joining during V(D)J recombination. To test whether inhibition of DNA-PKcs activity further impacts CSR in XLF-deficient cells, we activated WT or XLF<sup>Δ/Δ</sup> primary B cells for IgH CSR to IgG1 by culturing them in the presence of anti-CD40 plus IL-4, in the presence or absence of two different DNA-PKcs inhibitors (Nu-7026 and Nu-7441). In this context, treatment of WT-activated B cells had no significant effect on CSR to IgG1 in WT cells (Fig. 2E and Fig. S1), as previously observed (8). XLF-deficient B cells undergo CSR to IgG1 at about 30% the level of WT B cells at day 3,







indirect functionally redundant roles could be imagined in the context of proposed overlapping activities of ATM and DNA-PKcs (8, 16, 23). Whatever their redundant functions with XLF, DNA-PKcs and ATM have distinct roles, as neither can substitute for XLF in the absence of the other.

## Materials and Methods

**Mice.** All experiments involving mice were performed according to protocols approved by the Animal Resources Care Facility of Boston Children's Hospital (ARCH). Artemis<sup>+/-</sup> (40), DNA-PKcs<sup>+/-</sup> (15), Eμ-Bcl2<sup>+</sup> (41), Ku70<sup>+/-</sup> (42), XLF<sup>+Δ</sup> (29), and XRCC4<sup>ctt</sup> (43) mice were previously described.

**Chromosomal V(D)J Recombination Assays.** V(D)J recombination with an integrated substrate was previously described (20, 31, 32). Where indicated, the cells were treated with 15 μM of ATM inhibitor Ku-55933 (Tocris Bioscience) or 5 μM of Nu-7441 DNA-PKcs inhibitor (Tocris Bioscience). XLF<sup>Δ</sup> XRCC4<sup>ctt</sup> v-Abl clones with independent V(D)J cassette single integrations were treated with Tat-Cre and screened for *Xrcc4*<sup>+/-</sup> subclones as described (31, 44).

**Class Switch Recombination Assay.** Assays for IgH class switch recombination to IgG1 were carried out as previously described (37). In brief, mature B cells were isolated and pretreated with either DMSO vehicle or 5 μM of Nu-7026, or 5 μM of Nu-7441 (Tocris) and stimulated with anti-CD40 plus IL-4. Cells were assessed for switching to IgG1 by fluorescence activated cell sorting on day 4 as described.

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