CELL CYCLE
2017, VOL. 16, NO. 3, 286–295
http://dx.doi.org/10.1080/15384101.2016.1253640

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REPORT

Deficiency of XLF and PAXX prevents DNA double-strand break repair by non-homologous end joining in lymphocytes

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ABSTRACT

Non-homologous end joining (NHEJ) is a major DNA double-strand break (DSB) repair pathway that functions in all phases of the cell cycle. NHEJ repairs genotoxic and physiological DSBs, such as those generated by ionizing radiation and during V(D)J recombination at antigen receptor loci, respectively. DNA end joining by NHEJ relies on the core factors Ku70, Ku80, XRCC4, and DNA Ligase IV. Additional proteins also play important roles in NHEJ. The XRCC4-like factor (XLF) participates in NHEJ through its interaction with XRCC4, and XLF deficiency in humans leads to immunodeficiency and increased sensitivity to ionizing radiation. However, XLF is dispensable for NHEJ-mediated DSB repair during V(D)J recombination in murine lymphocytes, where it may have redundant functions with other DSB repair factors. Paralog of XRCC4 and XLF (PAXX) is a recently identified NHEJ factor that has structural similarity to XRCC4 and XLF. Here we show that PAXX is also dispensable for NHEJ during V(D)J recombination and during the repair of genotoxic DSBs in lymphocytes. However, a combined deficiency of PAXX and XLF blocks NHEJ with a severity comparable to that observed in DNA Ligase IV-deficient cells. Similar to XLF, PAXX interacts with Ku through its C-terminal region, and mutations that disrupt Ku binding prevent PAXX from promoting NHEJ in XLF-deficient lymphocytes. Our findings suggest that the PAXX and XLF proteins may have redundant functions during NHEJ.

ARTICLE HISTORY
Received 31 July 2016
Revised 19 October 2016
Accepted 22 October 2016

KEYWORDS
double-strand break repair; non-homologous end joining; PAXX; Pre-B cells; RAG; V(D)J recombination; XLF

Introduction

DNA double-strand breaks (DSBs) are generally repaired by homologous recombination (HR) or non-homologous end joining (NHEJ).[1,2] HR functions to repair DSBs in the S and G2 phases of the cell cycle using the sister chromatid as a template for precise restoration of DNA sequences.[2] NHEJ functions in all phases of the cell cycle and is the primary pathway of DSB repair in G1-phase cells. NHEJ rejoins broken DNA ends in a manner that is often imprecise, with the loss and gain of nucleotides at the join.[1,2] The core factors that are absolutely required for NHEJ in all settings include Ku70, Ku80, DNA Ligase IV, and XRCC4.[1,2] In addition, several other factors participate in NHEJ that may have redundant or more specialized functions.[1-4]

In developing lymphocytes, antigen receptor gene assembly occurs through the process of V(D)J recombination.[5,6] The recombination activating gene (RAG) endonuclease, composed of RAG1 and RAG2 proteins, recognizes recombination signal sequences (RSs) and generates DSBs at the border of RSs and the flanking recombining V (variable), D (diversity) or J (joining) gene segments.[6] RAG cleavage leads to the formation of a pair of hairpin-sealed coding ends (CEs) and a pair of blunt signal ends (SEs). The two coding ends are joined to form a coding join (CJ), and the 2 signal ends are joined to form a signal join (SJ).[7] In addition to the core NHEJ proteins, this joining requires other factors including the Artemis endonuclease, which opens hairpin-sealed CEs before they can be joined.[1,7] Artemis activity depends on the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs).[1,7] Thus, cells deficient in either Artemis or DNA-PKcs exhibit a nearly complete block in CJ formation but no defect in SJ formation.[1,7] The ataxia–telangiectasia mutated (ATM) kinase also functions in the repair of RAG DSBs, in part by promoting the stability of the broken DNA ends generated by RAG cleavage in a complex before they are joined by NHEJ.[8] Although neither ATM nor DNA-PKcs appear to be essential for SJ formation, a combined deficiency of these proteins leads to a nearly complete block in this process.[9,10] Thus, ATM and DNA-PKcs have overlapping activities that are critical for NHEJ-mediated DSB repair.

The XRCC4-like factor (XLF) functions during NHEJ in non-lymphoid cells, including the repair of RAG DSBs generated in plasmid substrates after transient expression of RAG.[11-16] XLF binds to the Ku heterodimer and to XRCC4, 2 core NHEJ factors, and forms filaments with XRCC4 that stabilize broken

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DNA ends prior to joining.[14,17-22] Paradoxically, in lymphoid cells there is a minimal requirement for XLF during RAG DSB repair by NHEJ.[12] However, a combined deficiency of XLF and either H2AX or 53BP1 leads to a block in DSB repair in lymphoid cells even though the singular loss of any of these proteins does not lead to notable NHEJ defects.[23-25] Moreover, a combined loss of XLF and either the DNA-PKcs or ATM kinase leads to a more significant block in NHEJ-mediated RAG DSB repair than a deficiency of any of these factors alone.[24,26] Finally, when combined with XLF deficiency, expression of a C-terminally truncated form of RAG2 also leads to a block in NHEJ.[27] Although XLF could have overlapping activities with several of these functionally diverse factors, it seems likely that many of these factors will have distinct activities, which when compromised in the setting of XLF deficiency, leads to notable defects in NHEJ-mediated DSB repair.

The paralog of XLF and XRCC4 (PAXX) was recently discovered based on its structural similarity with XLF and XRCC4 and on its association with other DNA repair factors.[28-30] XLF and PAXX bind each other and both bind to Ku70/Ku80 and XRCC4-DNA Ligase IV, suggesting that these proteins may be in a single complex.[28-30] Like XLF, PAXX deficiency leads to defects in NHEJ in non-lymphoid cells.[28-30] Here we use an approach to assay chromosomal V(D)J recombination in murine pre-B cells after RAG expression is induced.[8] We show that while deficiencies in either XLF or PAXX do not lead to defects in RAG DSB repair in pre-B cells, the combined deficiency of XLF and PAXX blocks the repair of RAG and genotoxic DSBs with a severity similar to that observed in DNA Ligase IV-deficient cells. These findings support the notion that XLF and PAXX have redundant NHEJ activities.

Results

Analysis of V(D)J recombination in abl pre-B cells: We have generated Abelson murine leukemia virus transformed pre-B cell lines, hereafter referred to as abl pre-B cells.[8] Treatment of abl pre-B cells with the v-Abl kinase inhibitor, imatinib, leads to G1 cell cycle arrest, the induction of RAG, and V(D)J recombination at the immunoglobulin kappa light chain (*Igk*) locus and within chromosomally integrated retroviral recombination substrates.[8] We generated a new retroviral recombination substrate, pMSCV-RSS-GFP-INV (hereafter pMGINV), that exhibits more stable expression than previous substrates (Fig. 1A).[8] pMGINV has an IRES-Thy1.2 cDNA that serves as a marker of infected cells (Fig. 1A). It also contains an antisense GFP cDNA flanked by RSs that mediate rearrangement by inversion, which places the GFP cDNA in the sense orientation leading to GFP expression (Fig. 1A).

pMGINV was introduced into both wild type (*WT:MGINV*) and DNA Ligase IV-deficient (*LigIV*$^{-/-}$*:MGINV*) abl pre-B cells. Treatment of these cells with imatinib leads to the generation of RAG DSBs at pMGINV in both *WT:MGINV* and *LigIV*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 1B-E). In *WT:MGINV* abl pre-B cells, these RAG DSBs are normally repaired, forming a CJ and a SJ and leading to GFP expression (Fig. 1A and B). Southern blotting reveals robust SJ formation in *WT:MGINV* abl pre-B cells, as indicated by the 2.4 kb *NheI* Thy1 probe hybridizing fragment after RAG induction (Fig. 1A and C). Hybridization of *XbaI* digested *WT:MGINV* genomic DNA with the GFP (Fig. 1D) and Thy 1 (Fig. 1E) probes failed to reveal 1 kb and 1.4 kb fragments, respectively, indicative of unrepaired CEs and SEs (Fig. 1A, D and E). Induction of RAG in *LigIV*$^{-/-}$*:MGINV* abl pre-B cells did not lead to the GFP expression due to the requirement for DNA Ligase IV to repair RAG DSBs (Fig. 1B). Indeed, Southern blot analysis of genomic DNA from *LigIV*$^{-/-}$*:MGINV* abl pre-B cells after RAG induction revealed hybridizing fragments generated by unrepaired SEs and CEs (Fig. 1A, D and E) and failed to reveal hybridizing fragments indicative of SJ formation (Fig. 1A and C). Together, these data establish that the pMGINV retroviral recombination substrate can be used to assay RAG cleavage and NHEJ-mediated DSB repair during V(D)J recombination in abl pre-B cells.

Generation of Xlf- *and* Paxx*-deficient abl pre-B cells*

Xlf$^{-/-}$ abl pre-B cells were generated from *Xlf*$^{-/-}$ mice that contained a Bcl-2 transgene.[24] A single copy of the pMGINV recombination substrate was introduced into these cells to generate *Xlf*$^{-/-}$*:MGINV* abl pre-B cells. CRISPR/Cas9 approaches were used to generate *Paxx*-deficient abl pre-B cells.[31] To this end, we introduced a lentiviral vector (pCW-Cas9) with a Cas9 cDNA under the control of a tetracycline-inducible promoter into *WT:MGINV* and *Xlf*$^{-/-}$*:MGINV* abl pre-B cells.[32] These cell lines exhibit robust induction of Cas9 upon doxycycline treatment (Fig. 2A). The lentiviral vector pKLV containing a guide RNA (gRNA) that targets the third exon of *Paxx* was introduced into *WT:MGINV* and *Xlf*$^{-/-}$*:MGINV* abl pre-B cells that have an integrated copy of pCW-Cas9.[33] After treatment with doxycycline and sub-cloning by limiting dilution, western blotting was used to identify *Paxx*$^{-/-}$*:MGINV* and *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 2B). Sequencing of the PAXX gene in the PAXX-deficient cells reveals deletions of various sizes (6 – 450 bp) in regions flanking the gRNA targeting sequence (Fig. S1).

Deficiencies in PAXX and XLF blocks NHEJ-mediated repair of RAG DSBs

To determine whether PAXX functions in the repair of RAG DSBs, we analyzed V(D)J recombination in *WT:MGINV* and *Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 3 and Fig. S2). PAXX deficiency did not impair rearrangement of pMGINV in *Paxx*$^{-/-}$*:MGINV* abl pre-B cells as compared to *WT:MGINV* abl pre-B cells (Fig. 3 and Fig. S2). In this regard, after induction of RAG with imatinib, the percentage of *Paxx*$^{-/-}$*:MGINV* abl pre-B cells expressing GFP (indicative of RAG DSB generation and repair) was similar to that observed in *WT:MGINV* abl pre-B cells (Fig. 3A). In agreement, Southern blot analyses revealed robust SJ formation (Fig. 3B and Fig. S2A) and no detectable unrepaired CEs or SEs (Fig. 3C, D and Fig S2B) in *Paxx*$^{-/-}$*:MGINV* abl pre-B cells. As expected, analyses of *Xlf*$^{-/-}$*:MGINV* abl pre-B cells also did not reveal any defects in V(D)J recombination (Fig. 3 and Fig. S2). Thus, like XLF deficiency, PAXX deficiency in abl pre-B cells does not lead to defects in V(D)J recombination.

We next examined abl pre-B cells deficient in both XLF and PAXX (*Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV*) (Fig. 3 and Fig. S2). In striking contrast to *Paxx*$^{-/-}$*:MGINV* and *Xlf*$^{-/-}$*:MGINV* abl pre-B cells,

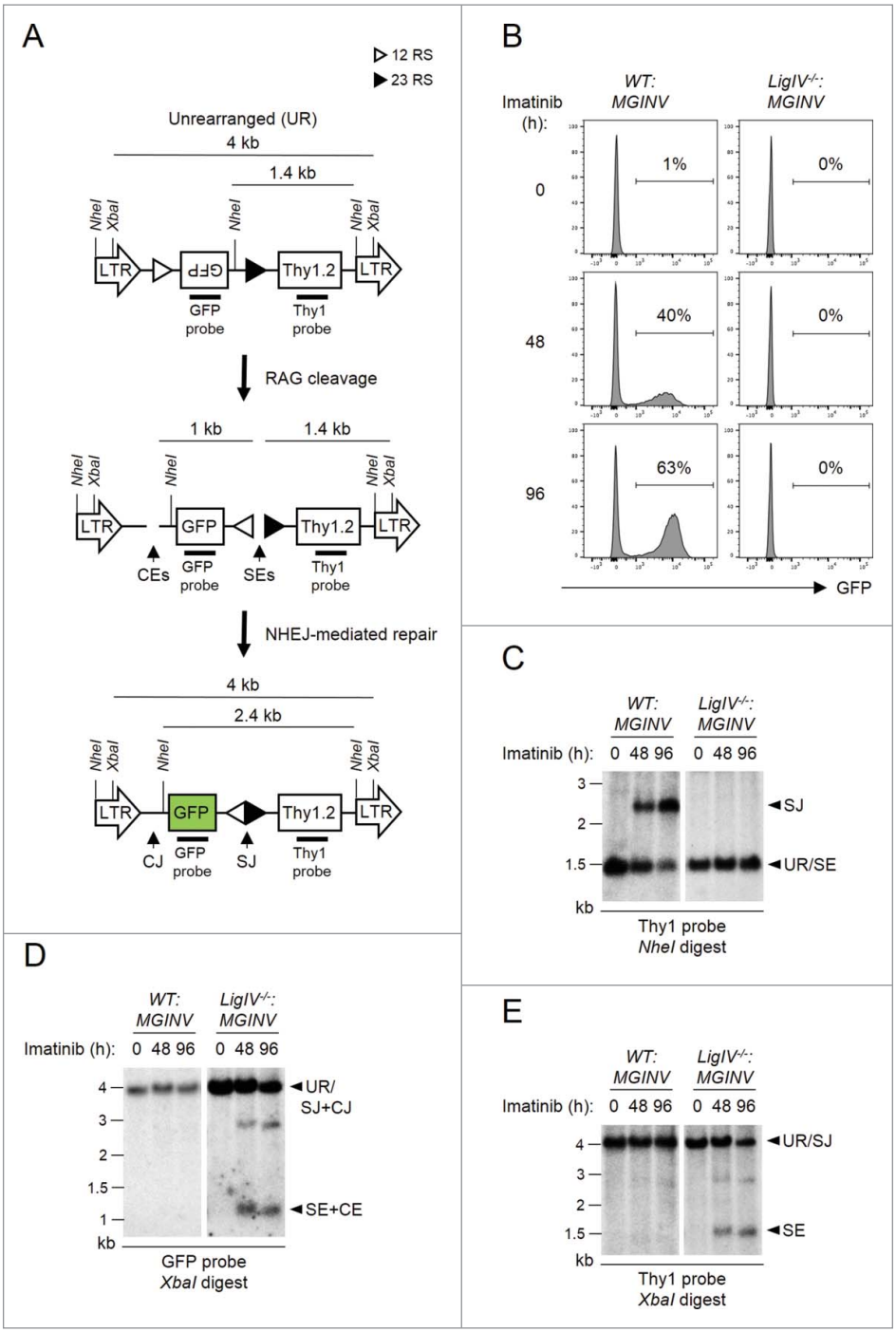

Figure 1. Rearrangement of pMGINV in abl pre-(B)cells. (A) Schematic of unrearranged (UR) pMGINV, its signal end (SE) and coding end (CE) intermediates following RAG cleavage, and the resulting signal join (SJ) and coding join (CJ) products. The long-terminal repeats (LTR), GFP cDNA, Thy1.2 cDNA, *NheI* and *XbaI* sites, recombination signals (RSs, represented by open and filled triangles), and Thy1 and GFP probes are shown. (B) *WT:MGINV* and *LigIV*$^{-/-}$*:MGINV* abl pre-B cells were treated with imatinib for 0, 48, or 96 hours (h). The percentage of GFP-positive cells is indicated. (C-E) *WT:MGINV* and *LigIV*$^{-/-}$*:MGINV* abl pre-B cells were treated with imatinib for the indicated times (h), and Southern blot analyses were carried out on *NheI*-digested (C) or *XbaI*-digested (D, E) genomic DNA isolated from these cells and hybridized with the Thy1 (C, E) or GFP (D) probe. Hybridizing bands from unrearranged pMGINV (UR), SJs, SEs, both CJs and SJs (SJ+CJ) and both SEs and CEs (SE+CE) are indicated. Molecular weight markers (kB) are also indicated.

Xlf$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells exhibit a dramatic defect in V(D)J recombination, with very few cells expressing GFP after RAG induction with imatinib (Fig. 3A). This is due to a block in the repair of RAG DSBs, as evidenced by Southern blot analyses that reveal very little SJ formation and an accumulation of unrepaired CEs and SEs similar in magnitude to that observed in *LigIV*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 3B-D, Fig. 1D, E and Fig. S2). We conclude that the combined deficiency in XLF and PAXX leads to a defect in NHEJ of similar severity to that observed in DNA Ligase IV-deficient abl pre-B cells.

Defective genotoxic DSB repair in Paxx$^{-/-}$:Xlf$^{-/-}$ *abl pre-B cells*

In non-lymphoid cells, PAXX and XLF are both required for the repair of DSBs generated by genotoxic agents.[11-16, 28-30] To

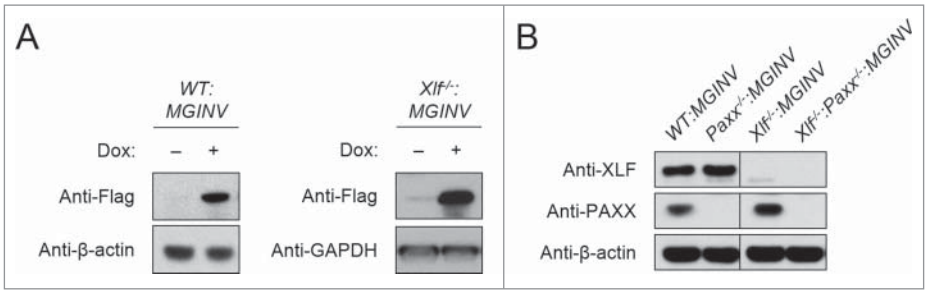

Figure 2. Generation of *Paxx*$^{-/-}$ and *Xlf*$^{-/-}$:*Paxx*$^{-/-}$ abl pre-(B)cells: (A) Western blot of lysates from *WT:MGINV* and *Xlf*$^{-/-}$*:MGINV* abl pre-B cells stably transduced with pCW-Cas9 in the absence (−) and presence (+) of doxycycline. Western blots were probed with antibodies to Flag (to detect Flag-Cas9), with β-actin or GAPDH used as protein loading controls. (B) Western blotting for XLF, PAXX, and β-actin in lysates from *WT:MGINV, Xlf*$^{-/-}$*:MGINV, Paxx*$^{-/-}$*:MGINV* and *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells.

assess the requirement for PAXX and XLF in repairing genotoxic DSBs in lymphocytes, we monitored nuclear foci of phosphorylated H2AX (γH2AX) in G1-arrested *WT:MGINV, Xlf*$^{-/-}$*:MGINV, Paxx*$^{-/-}$*:MGINV, Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* and *LigIV*$^{-/-}$*:MGINV* abl pre-B cells after treatment with bleocin, a radiomimetic agent (Fig. 4A and B). γH2AX forms when DNA damage response kinases, such as ATM and DNA-PKcs, phosphorylate H2AX in chromatin flanking DNA DSBs, and γH2AX is lost after DSB repair. After two hours of bleocin treatment, 35% to 45% of all cell lines examined had greater than 5 γH2AX foci (Fig. 4A and B). When cells were removed from bleocin treatment, most of the γH2AX foci resolved by 24 hours in *WT:MGINV, Xlf*$^{-/-}$*:MGINV* and *Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 4A and B). However, as is observed in *LigIV*$^{-/-}$*:MGINV* abl pre-B cells, which have a complete block in NHEJ, the percentage of *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* cells with more than 5 γH2AX foci remains unchanged, indicative of persistent unrepaired DSBs (Fig. 4A and B). Similarly, *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* cells were also unable to resolve γH2AX foci induced by etoposide, which induces DSBs through a mechanism distinct of bleocin (Fig. 4C and D). Thus, while there are no detectable defects in the repair of genotoxic DSBs in abl pre-B cells with isolated deficiencies in either XLF or PAXX, combined deficiency of XLF and PAXX in abl pre-B cells leads to a

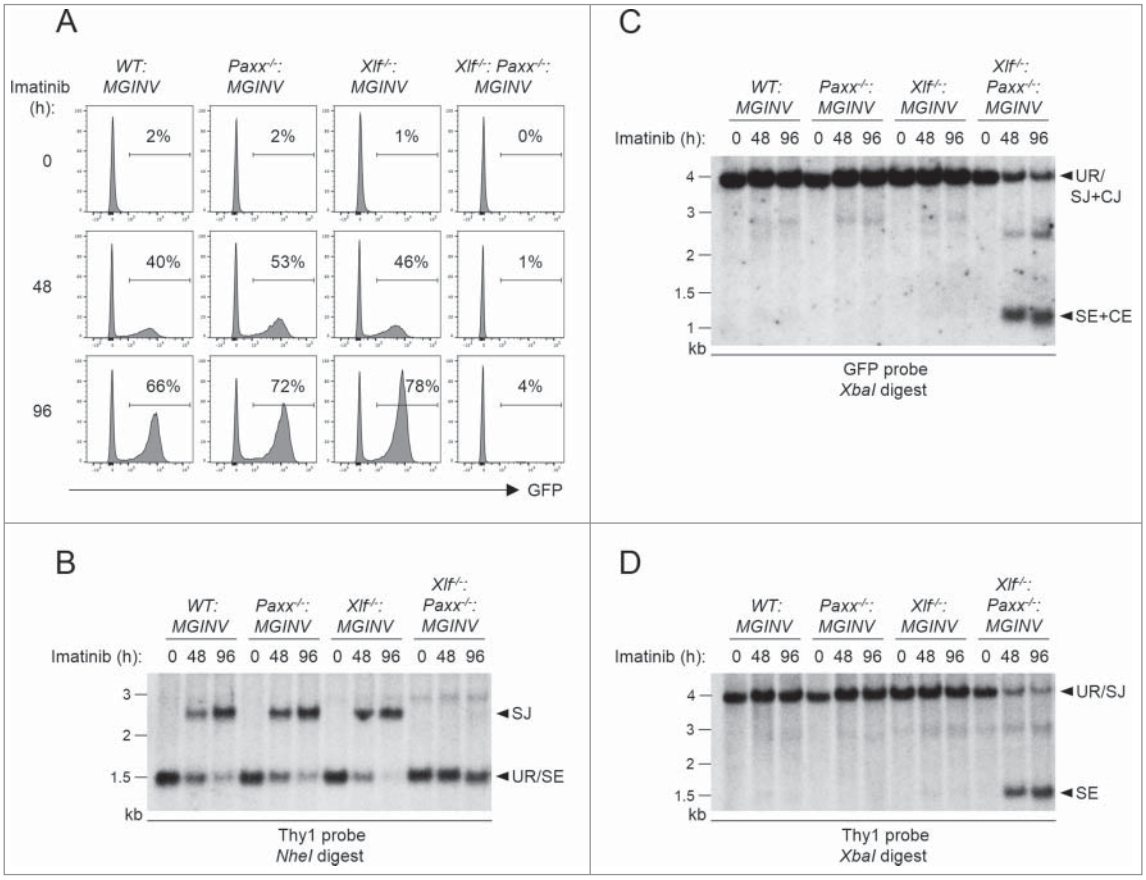

Figure 3. RAG DSB repair in PAXX- and XLF-deficient abl pre-(B)cells: (A) *WT:MGINV, Xlf*$^{-/-}$*:MGINV, Paxx*$^{-/-}$*:MGINV* and *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells were treated with imatinib for 0, 48, or 96 hours (h). The percentage of GFP-positive cells is indicated. (B-D) Southern blot analyses of *NheI*-digested (B) or *XbaI*-digested (C, D) genomic DNA isolated from imatinib-treated abl pre-B cells in (A) and hybridized with the Thy1 (B, D) or GFP (C) probe. Hybridizing bands for different pMGINV rearrangements are indicated as described in Fig. 1A. Molecular weight markers are indicated (kb).

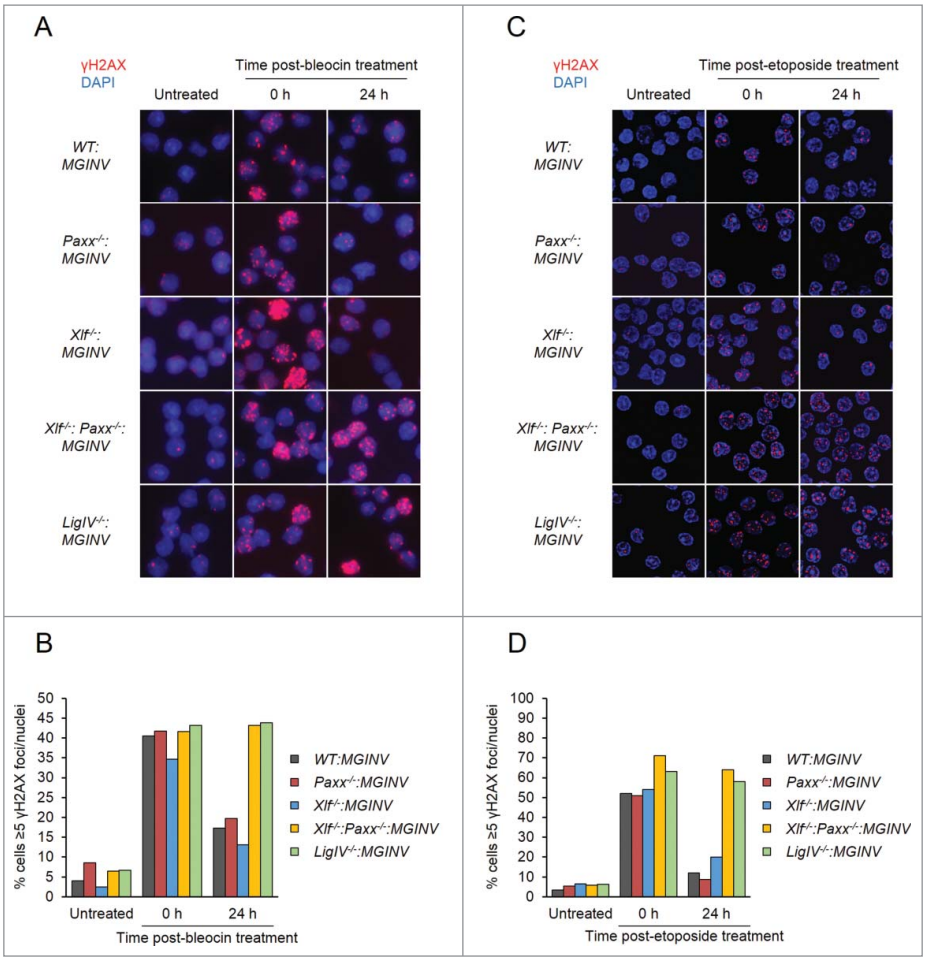

Figure 4. Genotoxic DSB repair in PAXX- and XLF-deficient abl pre-(B)cells: (A, C) Representative micrographs of γH2AX foci in *WT:MGINV, Paxx*$^{-/-}$*:MGINV, Xlf*$^{-/-}$*:MGINV, Xlf*$^{-/-}$*: Paxx*$^{-/-}$*:MGINV*, and *LigIV*$^{-/-}$*:MGINV* abl pre-B cells that were untreated or treated with bleocin (A) or etoposide (C) and then allowed to recover in fresh media for 0 or 24 hours. (B, D) Quantitation of cells in (A) or (C) with ≥5 γH2AX foci per nucleus. At least 100 nuclei were scored for each condition. Data shown is representative of 3 replicate experiments.

defect in the repair of genotoxic DSBs as severe as that observed in DNA Ligase IV-deficient abl pre-B cells.

The PAXX C-terminal domain is required for RAG DSB repair

PAXX is composed of a N-terminal globular head domain, a central coiled-coil region and a low complexity C-terminal domain (Fig. 5A). This structural arrangement is similar to that of XRCC4 and XLF.[14,28-30,34] XLF and XRCC4 associate with one another through their N-terminal domains, and their C-terminal domains are known to interact with many DSB repair proteins.[14,17,35-39] While there is no known function for the PAXX N-terminal head domain, mutations of this domain in human PAXX have been shown to effect its function in DSB repair in non-lymphoid cells.[29] The C-terminal region of PAXX binds to Ku and mutations that disrupt this binding also effect the function of human PAXX in NHEJ.[28,29] To determine whether these 2 domains are also critical for the repair of RAG DSBs in lymphocytes, we ectopically expressed wild type or mutant PAXX proteins in *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 5A and B). Expression of wild type PAXX, which interacts with Ku70 (Fig. 5C), rescued the repair of RAG DSBs to nearly normal levels in *Xlf−/−:Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 5D-F). In contrast, PAXX^{VF}, in which the evolutionarily conserved valine and phenylalanine in the C-terminal Ku-binding motif have been mutated to alanine (V200A and F202A), failed to bind Ku70 (Fig. 5A and C) and was unable to rescue V(D)J recombination (Fig. 5D–F). PAXX^{Nmut}, in which we mutated the 4 conserved leucine and isoleucine residues (I98D, L100D, L107D and L111D) in the N-terminal head domain previously mutated in human PAXX, was also unable to rescue V(D)J recombination in *Xlf*$^{-/-}$*:Paxx*$^{-/-}$*:MGINV* abl pre-B cells (Fig. 5A and D-F).[29] However, the PAXX^{Nmut} mutations appear to destabilize the murine PAXX protein leading to low levels of PAXX^{Nmut} expression (Fig. 5B and C). Thus, it is not possible for us to make any conclusions about requirements for the N-terminus of PAXX in mediating NHEJ in murine lymphocytes. However, these findings clearly implicate a function for the Ku-binding C-terminal domain of PAXX in the NHEJ-mediated repair of RAG DSBs in murine lymphocytes.

Discussion

The XLF protein is required for normal NHEJ in non-lymphoid cells but is dispensable for NHEJ in lymphocytes. PAXX also functions in NHEJ in non-lymphoid cells, and here we show that, like XLF, PAXX is also dispensable for NHEJ in lymphoid

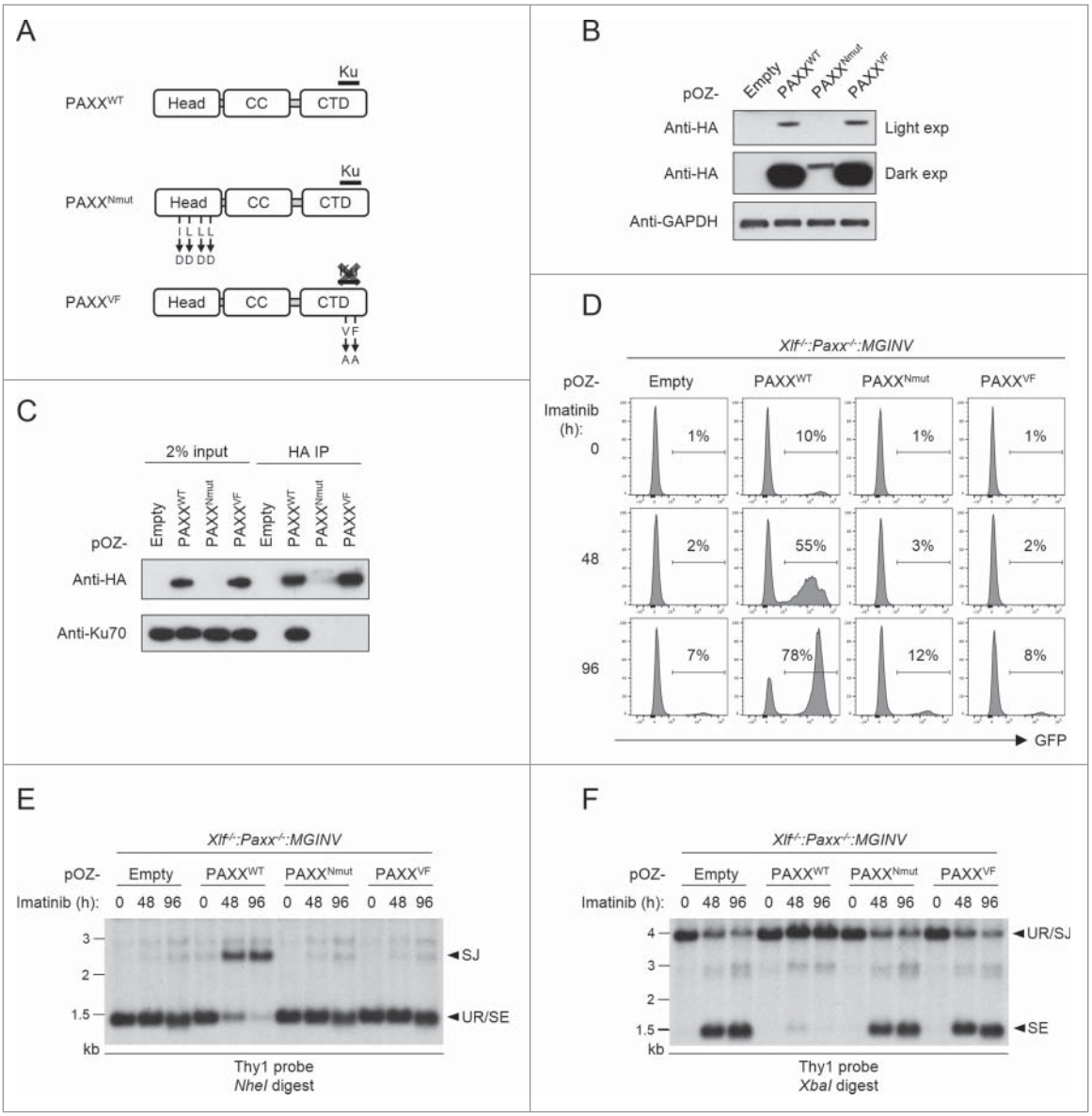

Figure 5. Function of PAXX mutants in NHEJ: (A) The 3 major domains of the PAXX protein: N-terminal head region, coiled coil domain (CC) and C-terminal domain (CTD). The mutations in the are PAXX^{Nmut}, and PAXX^{VF} also specified. (B) Western blot analysis of lysates from *Xlf*$^{-/-}$:*Paxx*$^{-/-}$:*MGINV* abl pre-B cells reconstituted with Flag-HA-tagged PAXX^{WT}, PAXX^{Nmut}, and PAXX^{VF}. Antibodies to HA (Paxx) and GAPDH were used. (C) Western blot analysis of Ku70 association with Paxx and Paxx mutants in *WT:MGINV* abl pre-B cells. *WT:MGINV* abl pre-B cells expressing Flag-HA-tagged PAXX^{WT}, PAXX^{Nmut}, and PAXX^{VF} were subjected to immunoprecipitation using anti-HA followed by western blotting with anti-HA (Paxx) or anti-Ku70. Input lysates are also shown. (D) *Xlf*$^{-/-}$:*Paxx*$^{-/-}$:*MGINV* abl pre-B cells expressing PAXX^{WT}, PAXX^{Nmut}, and PAXX^{VF} were treated with imatinib for 0, 48, or 96 hours (h). The percentage of GFP-positive cells is indicated. (E, F) Southern blot analyses of *NheI*-digested (E) and *XbaI*-digested (F) genomic DNA isolated from imatinib-treated abl pre-B cells in (D) and hybridized with the Thy1 probe. Hybridizing bands for different pMGINV rearrangements are shown as described in Fig. 1A. Molecular weight markers are indicated (kb).

cells. NHEJ-mediated repair of RAG DSBs and DNA DSBs generated by the radiomimetic agent bleocin is unperturbed in PAXX-deficient abl pre-B cells. However, the combined deficiency of XLF and PAXX causes a dramatic block in the NHEJ-mediated repair of both RAG and genotoxic DSBs in abl pre-B cells. The defect in NHEJ-mediated DSB repair in *Xlf*$^{-/-}$:*Paxx*$^{-/-}$:*MGINV* cells is as profound as the defect observed in DNA Ligase IV-deficient abl pre-B cells. During the revision of this manuscript 2 papers were published that also demonstrate that combined deficiency in PAXX and XLF leads to a significant block in the repair of RAG DSBs.[40,41]

In lymphoid cells, combined deficiency of XLF and several other DNA damage response factors also results in defects in NHEJ that are more significant than their isolated deficiencies. This includes the DNA damage response kinases ATM and DNA-PKcs, the histone variant H2AX, and the DNA repair protein 53BP1.[23-26] These proteins could have redundant functions with XLF in NHEJ-mediated DSB repair, or perhaps XLF deficiency compromises NHEJ in lymphoid cells in a manner that is undetectable in the absence of additional, mechanistically distinct, defects. In this regard, both H2AX and 53BP1 function to protect broken DNA ends from being resected and forming single-strand DNA overhangs that would inhibit NHEJ.[42-46] Thus, if XLF deficiency slows the kinetics of NHEJ, it is possible that the concomitant deficiency of either H2AX or 53BP1 could block repair by allowing broken DNA ends to be

resected before they can be joined.[23-25] Notably, signal and coding ends in abl pre-B cells deficient in XLF and PAXX do not appear to be resected, making it less likely that PAXX deficiency leads to a block in NHEJ due to a requirement for PAXX in protecting broken DNA ends.

PAXX may function in NHEJ in a way that is mechanistically distinct from XLF. However, XLF and PAXX have very similar structures, raising the possibility that they may have overlapping functions. PAXX may exist as homodimer, which could potentially form high-order filaments similar to those formed by XLF-XRCC4 heterodimers that have been implicated in tethering and stabilizing DNA ends prior to repair.[28] In addition, PAXX and XLF also both interact with the Ku heterodimer through their C-terminal regions, and their localization to laser-induced damage sites depends on such interaction.[17,28,29,47] PAXX exhibits more stable binding to Ku *in vitro* when Ku is bound to DNA.[48] While XLF and PAXX show little or no DNA binding affinity themselves, they stimulate *in vitro* DNA ligation in a Ku-dependent manner, suggesting that they may act as a scaffold through interaction with Ku to stabilize the XRCC4-DNA Ligase IV complex.[28,49] By extension, *in vivo*, they could both promote the stable assembly of similar sets of repair factors. In this regard, a recent study demonstrated that PAXX-deficient human cells exhibit a substantial defect in the recruitment of several repair proteins to DNA DSBs.[28] Whether XLF deficiency leads to the same defect in the recruitment of these factors is not known, and it will be interesting to determine if the combined deficiency of XLF and PAXX results in a more severe defect in the recruitment of these factors to DNA DSBs.

Why is it that NHEJ is compromised in non-lymphoid cells with isolated deficiencies of PAXX or XLF, but in lymphoid cells, defects in NHEJ are observed only when there is a combined deficiency of these proteins? It is possible that while PAXX and XLF have generally overlapping activities in NHEJ, they may also have unique functions in non-lymphoid cells that are not required for NHEJ in lymphoid cells. PAXX and XLF could exist in a complex with each other and several other key NHEJ proteins. The components of this complex could vary qualitatively or quantitatively in a way that leads to independent requirements for PAXX and XLF during NHEJ in different tissues.

Materials and methods

Generation of pMGINV

To generate pMGINV (pMSCV-RSS-GFP-INV), the murine J_k5 RS was cloned into the *ClaI* site of pBluescript, and the V_k8 RS was cloned into the resulting vector between the *EcoRV* and *NotI* sites. The *ClaI* and *NotI* sites were destroyed. The RSs were oriented as direct repeats, so that rearrangement between them occurs by inversion. The GFP cDNA was then blunt cloned into the *EcoRV* site between the 2 RSs. The RS-GFP cassette was removed from pBluescript using *SalI* and *SacII*, then blunt cloned into a *BglII* site upstream of IRES-Thy1.2 in pMSCV-IRES-Thy1.2, with the GFP cDNA in the antisense orientation. The *BglII* site was recreated upstream of the RS-GFP cassette, but was destroyed downstream, leaving a unique *BglII* site.

Cell line generation and cell culture

Wild type and $Xlf^{-/-}$ abl pre-B cells were created as previously described.[8,12] Briefly, bone marrow cells were harvested from 3–5 week-old mice harboring the Eμ-Bcl2 transgene and infected with the pMSCV-v-abl retrovirus to generate stable v-abl-transformed pre-B cell lines. These cells were sequentially transduced with pMGINV and pCW-Cas9 (Plasmid #50661, Addgene) and selected for Thy1.2 expression and puromycin resistance before sub-cloning by limiting dilution. Clones were treated with 2 μg/mL doxycycline for 2 d and assayed for Cas9 induction by western blotting. *WT:MGINV* and $Xlf^{-/-}$*:MGINV* abl pre-B cells with highly inducible Cas9 expression were further transduced with a lentiviral vector pKLV (Plasmid #50946, Addgene) containing a gRNA that targets the mouse *Paxx* coding sequence (5′- AGATATCCATTCCCGGTTC-3′), sorted for BFP expression, and treated with doxycycline for a week before sub-cloning by limiting dilution. $Paxx^{-/-}$*:MGINV* and $Xlf^{-/-}$*:*$Paxx^{-/-}$*:MGINV* cells were screened by western blotting. All experiments were repeated in 2 knockout cell lines. Abl pre-B cells were cultured in media with 3 μM imatinib (Novartis) at a density of 10^6 cells/mL to induce G1 arrest.

Mutated *Paxx* alleles were amplified with primers PAXX 5′ intron 2S (GTGAGTA ACAGTGCTGGGGATA) and PAXX 3′ intron 3 AS (CTAAGGAGGGAGATGTGT GTTA), with the exception of PCR reaction for $Xlf^{-/-}$*:*$Paxx^{-/-}$*:MGINV* clone 5, where the PAXX 3′ intron AS primer was replaced with PAXX 3′ *NotI*. PCR products were cloned to pCRII-TOPO vector and sequences determined by M13 (Reverse) primer.

Protein analyses

Western blotting was performed as previously described.[42] The primary antibodies used for western blotting and immunoprecipitation were: mouse anti-Flag (F3165, Sigma), rabbit anti-XLF (A300-729A, Bethyl Laboratories), rabbit anti-PAXX (ab126353, Abcam), rabbit anti-HA [Y-11] (sc805, Santa Cruz), rabbit anti-cytoskeletal actin (A300-491A, Bethyl Laboratories), and mouse anti-GAPDH (G8795, Sigma). Immunoprecipitation (IP) was performed as described in Ciccia et al.[50] Cell lysate extracted from 30 million G1-arrested wild type abl pre-B cells transduced with pOZ-FH-$Paxx^{WT}$, $Paxx^{Nmut}$, or $Paxx^{VF}$ were used for each IP with 5 μg of mouse anti-HA IgG.

Southern blot analysis

Southern blotting were performed as previously described.[8] The Thy1 probe is a ~800-bp Thy1.1 cDNA fragment, while the GFP probe is a ~700-bp GFP cDNA fragment.

Generation of Paxx mutants

The wild type *Paxx* coding sequence was amplified from cDNA clone BC029124 with primers PAXX 5′ *XhoI* (5′-GCCCTCG AGATGGCTCCTCCGTTGTTGTC-3′) and PAXX 3′ *NotI* (5′-GCCGCGGCCGCT CAGGTCTCATCAAAGTCTA-3′) To ge

nerate PAXX^{Nmut}, primers PAXX 5′ *XhoI* and PAXX_NmutAS (5′-GGAG**TC**GTCAAAGGC**GTC**GGCAGGGGTATCCCCTG AA**TC**GGT**ATC**CAATGC-3′) were used to generate the 5′ Nmut fragment. Primers PAXX 3′ *NotI* and PAXX_ NumtS (5′-GCATTG**GAT**ACC**GA**TTCAGGGG ATACCCCTGCC-**GAC**GCCTTTG AC**GA**CTCC-3′) were used to generate the 3′ Nmut fragment. The mutated sequences for PAXX^{Nmut} and PAXX^{VF} (below) are highlighted in bold. Overlapping PCR was used to convert the 2 fragments to full-length PAXX^{Nmut}. To generate PAXX^{VF} mutant, primers PAXX 5′ *XhoI* and PAXX_V,F-A_3′ *NotI* (5′-GCCGCGGCCGCTCAGGTCTCA TCA**GC**GTCT**G**CAC CAGCAGCTGGT-3′) were used in PCR to incorporate mutated sequences in the *Paxx* coding sequences (bold fonts in the primer PAXX_V,F-A_3′ *NotI*). The resulting DNA fragments *Paxx*^{WT}, *Paxx*^{Nmut} and *Paxx*^{VF} were cloned into *XhoI* and *NotI* sites of the retroviral vector pOZ-Flag-HA-N (pOZ-FH-N).

Nuclear foci

Abl pre-B cells were cultured in media with 3 μM imatinib for 2 d to induce G1 arrest and then treated with 10 μg/mL bleocin (203408, Millipore) for 2 hours, or 2 ug/mL etoposide (E1383, Sigma) for 1 hour, after which the cells were washed in PBS and rested in fresh imatinib media for 24 hours. For immunofluorescent staining of γH2AX, abl pre-B cells were plated onto glass coverslips coated with Cell-Tak (354240, Corning), fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, blocked with 3% BSA/PBS (w/v) for 1 hour at room temperature, and incubated with mouse anti-γH2AX (05–636 Millipore) at a 1:1,000 dilution overnight at 4°C. The coverslips were then washed in PBS, incubated with donkey anti-mouse IgG (H + L) Alexa Fluor 594 at 1:1,000 dilution (A-21203, ThermoFisher) for 1 hour at room temperature, washed again in PBS, and mounted onto microscope slides (12-550-343, Fisher Scientific) in ProLong Gold Anti-Fade Mountant with DAPI (P36931, ThermoFisher). Images were acquired at 60X magnification using an Olympus BX53 fluorescence microscope.

Abbreviations

ATM	ataxia–telangiectasia mutated
CE	coding end
CJ	coding join
CRISPR	clustered regularly interspaced short palindromic repeats
DNAPKcs	DNA-dependent protein kinase catalytic subunit
DSB	double-strand breaks
HR	homologous recombination
NHEJ	non-homologous end joining
PAXX	paralog of XLF and XRCC4
pMGINV	pMSCV-RSS-GFP-INV
RAG	recombination activation gene
RS	recombination signal sequence
SE	signal end
SJ	signal join
V(D)J	variable diversity and joining
XLF	XRCC4-like factor

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest..

Acknowledgments

We thank Drs. John Petrini and Wytse Bruinsma for help carrying out foci experiments, and Dr. Frederick Alt for *XLF*$^{+/-}$ mice and Mr. Ryan Irwin for managing mouse colonies.

Funding

This work was in part supported by NIAID grant 7RO1AI074953 and NCI grant 5R01CA177759 to BPS, and NCI grant 5F32CA183271 to BC.

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