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Functional Overlaps Between XLF and The ATM-dependent DNA Double Strand Break Response

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Abstract

Developing B and T lymphocytes generate programmed DNA Double Strand Breaks (DSBs) during the V(D)J recombination process that assembles exons that encode the antigen-binding variable regions of antibodies. In addition, mature B lymphocytes generate programmed DSBs during the Immunoglobulin Heavy chain (IgH) Class Switch Recombination (CSR) process that allows expression of different antibody heavy chain constant regions that provide different effector functions. During both V(D)J recombination and CSR, DSB intermediates are sensed by the ATM-dependent DSB response (DSBR) pathway, which also contributes to their joining via Classical Non-Homologous End-Joining (C-NHEJ). The precise nature of the interplay between the DSBR and C-NHEJ pathways in the context of DSB repair via C-NHEJ remains under investigation. Recent studies have shown that the XLF C-NHEJ factor has functional redundancy with several members of the ATM-dependent DSBR pathway in C-NHEJ, highlighting unappreciated major roles for both XLF as well as the DSBR in V(D)J recombination, CSR and C-NHEJ in general. In this review, we discuss current knowledge of the mechanisms that contribute to the repair of DSBs generated during B lymphocyte development and activation with a focus on potential functionally redundant roles of XLF and ATM-dependent DSBR factors.

Keywords

Cernunnos; ATM; DNA-PKcs; NHEJ; V(D)J recombination; 53BP1

1. Repair of Programmed DSBs during Lymphocyte Development and Activation

Classical Non-Homologous End-Joining (C-NHEJ) is a major mammalian double-strand break (DSB) repair pathway that is active throughout the cell cycle but which has particular importance in the G1 cell cycle phase. There are two programmed DNA recombination

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processes in lymphocytes that employ C-NHEJ: V(D)J recombination in developing B and T lymphocytes, and IgH Class Switch Recombination (CSR) in activated mature B lymphocytes [1–4]. In addition to C-NHEJ, homologous recombination (HR) is the other known major DSB repair pathway in mammalian cells and predominates in the S/G2 phases of the cell cycle. Moreover, one or more Alternative End-Joining (A-EJ) DSB repair mechanisms have been observed to fuse at least certain classes of DSBs in the absence of C-NHEJ; however, the overall functional significance of A-EJ in the presence of C-NHEJ is yet to be determined [1, 2].

In developing B and T cell lymphocytes, variable (V), diversity (D) and joining (J) gene segments are assembled to form V(D)J exons that encode the N-terminal “variable” portion of immunoglobulin (Ig) or T cell receptor (TCR) proteins that provide antigen binding specificity. Together with downstream exons that encode C-terminal “constant” region portions of Ig or TCR proteins, they form complete Ig or TCR genes [5]. V(D)J recombination is initiated by the Recombination activating gene 1 and 2 proteins, which together form an endonuclease (RAG) that recognizes short recombination signal (RS) sequences that flank V, D, or J coding sequences [6] and then introduces DSBs between an appropriate set of RSs and V, D, or J coding gene segments [7, 8]. RAG generates DSBs at these sequences in the form of blunt 5′-phosphorylated signal RS ends (“SEs”) and hairpin-sealed coding ends (“CEs”) [9]. The RAG complex holds cleaved V(D)J CE and SEs in a post-cleavage synaptic complex and, in a yet to be determined manner, directs joining of two appropriate CEs to each other to form V(D)J coding joins (“CJs”) and joining of the two SEs to each other to form signal joins (“SJs”), by the C-NHEJ pathway [10] (Figure 1).

V(D)J recombination end-joining occurs exclusively by C-NHEJ and does not occur at all in the absence of core C-NHEJ factors [11]. In this regard, RAG2, through unknown mechanisms, functions to exclude potential A-EJ repair pathways [12]. Consistent with V(D)J joining occurring only by C-NHEJ, the entire V(D)J recombination reaction occurs only in the G1 cell cycle phase, a restriction solidified by the degradation of RAG2 at the G1/S transition [13, 14]. Overall, the “V(D)J recombinase” consists of the lymphocyte-specific RAG cleavage component and lymphocyte-specific TdT diversification component (see below), along with more generally expressed C-NHEJ proteins that comprise the joining component. As discussed below, recent studies also implicate ATM-dependent DSB repair factors as potential joining components of the V(D)J recombinase. It is notable that during V(D)J recombination, RAG must alter the normal process of C-NHEJ from simply rejoining the two ends of a given DSB to direct joins of CEs to CEs and SEs to SEs from two separate DSBs, leading to an inversional or deletional outcome depending on the relative chromosomal orientation of the initiating RSs to each other [13, 15, 16].

Mature B cells are generated through the productive assembly of an IgH V(D)J exon and an Ig light chain (IgL) VJ exon; which, respectively, lead to production of IgH and IgL proteins that together form the B cell antigen receptor (“BCR”). In the peripheral immune system (e.g. spleen and lymph nodes), mature B cells may encounter antigens that bind to their BCR, causing them to become activated and to undergo additional genomic alterations including IgH Class Switch Recombination (CSR). Through CSR, the set of exons encoding the initially expressed IgH constant region (C μ) are replaced with one of several sets of

downstream C_H exons (C_γ, C_α, C_ε), leading to IgH class switching from IgM to IgG, IgA, or IgE. CSR results in secretion of specific antibodies that are endowed with optimal effector functions for elimination of particular pathogens. CSR is initiated by the Activation-induced cytidine deaminase (AID), which deaminates C's to U's within long (i.e. up to 10kb), highly repetitive switch ("S") regions that lie just upstream of each set of C_H exons, thereby triggering downstream mechanisms that generate S region DSBs that are requisite intermediates for CSR [1, 2]. To complete CSR, DSBs in a donor S region upstream of C_μ(S_μ) and a downstream acceptor S region (e.g. S_γ, S_α, S_ε) are fused by C-NHEJ. However, in the complete absence of C-NHEJ, CSR, unlike V(D)J recombination, can still be completed, albeit at somewhat reduced efficiency, by A-EJ [2, 17–19].

2. C-NHEJ in the repair of DSB during V(D)J recombination and other processes

The Ku70, Ku80, XRCC4 and DNA Ligase 4 (Lig4) factors are often described as the evolutionarily conserved "core" C-NHEJ factors [1, 2, 20, 21]. Ku70 and Ku80 form a dimer ("Ku") that recognizes and binds to DSBs [22]. XRCC4 and Lig4 form a complex that is requisite for the ligation phase of C-NHEJ [1, 20, 21]; these proteins apparently are recruited to DSB ends by Ku [23, 24]. Due to their recognition and joining functions, respectively, Ku and XRCC4/Lig4 are required for all known forms of C-NHEJ; for example, during V(D)J recombination they are required for joining of blunt SEs and for joining of hairpin-sealed CE [1, 2]. Accordingly, deficiency for any of the core C-NHEJ factors (Ku70, Ku80, XRCC4 or Lig4) in mice leads to inability to join CE or SEs during V(D)J recombination, resulting in a complete block in B and T cell development and a Severe Combined Immunodeficiency (SCID), that is essentially as severe as that of RAG-deficient mice where V(D)J recombination cannot be initiated [2, 25, 26].

The DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis, which is a DNA-PKcs-activated endonuclease, are C-NHEJ factors that have specialized functions in C-NHEJ that are required for joining DNA ends that must be processed before joining. For example, joining of hairpin-sealed CE is nearly abrogated in the absence of DNA-PKcs or Artemis due to their inability to be opened in the absence of DNA-PKcs-activated Artemis endonuclease activity [27]. In accord with the requirement for DNA-PKcs and Artemis to generate V(D)J recombination CJs, complete deficiency for DNA-PKcs or Artemis in mice also results in a block in B and T cell development and a SCID due to inability to join V(D)J CE and thereby form functional Ig and TCR genes required for development beyond progenitor stages [2, 25]. Processing of DSBs for C-NHEJ also may involve various DNA polymerases, such as DNA polymerase μ and DNA polymerase λ [20]. In addition, the developing lymphocyte specific terminal deoxynucleotidyl transferase enzyme (TdT), the first recognized component of the V(D)J recombinase [28], adds non-templated nucleotides to V(D)J junctions prior to their ligation, thereby greatly increasing junctional diversity and vastly expanding the diversity of antibody and TCR repertoires [28–30]. Once DSB ends are processed, if necessary, the XRCC4/Lig4 complex ligates them to complete the reaction.

DNA-PKcs clearly has functions in C-NHEJ beyond activating Artemis. In this regard, ligation of blunt SEs occurs normally in the absence of Artemis; but SE joining is variably

impaired, depending on cell type, both with respect to frequency and fidelity in the absence of DNA-PKcs. Thus, SE joining occurs normally in DNA-PKcs-deficient embryonic stem (ES) cells but shows variable impairment in developing lymphocytes and somatic cell lines [31–38]. Among other possibilities, such variability in SE joining among different DNA-PKcs-deficient cell types might be explained by differential expression in different cell types of factors that are functionally redundant with DNA-PKcs C-NHEJ functions beyond those associated with Artemis activation. As discussed below, two known factors, XLF and ATM, can, at least partially, compensate for DNA-PKcs in SE joining. Non-Artemis-related C-NHEJ functions of DNA-PKcs revealed by defective SE joining during V(D)J recombination in DNA-PKcs-deficient cells have been suggested to include DNA end-bridging functions based on biochemical studies [39–41]. While DNA-PKcs may also play such end-bridging functions in C-NHEJ more generally, such a role is difficult to study in the context of V(D)J recombination CE joining due to requisite function of DNA-PKcs in the opening of hairpin-sealed CEs prior to joining.

C-NHEJ is involved in repair of DSBs more generally. Deficiency for core C-NHEJ factors in lymphoid or non-lymphoid cells leads to IR sensitivity and increased genomic instability, associated defects in repair of DSBs ranging from DSBs introduced by the I-SceI enzyme in reporter constructs [42, 43] to DSBs introduced during CSR in activated B cells [17–19, 42] and to DSBs resulting from unknown factors during neuronal development. In the latter context, deficiency of XRCC4 or Lig4 results in severe and widespread p53-mediated apoptotic death of newly generated neurons in response to DSBs [44–48]. This severe neuronal death is associated with late embryonic lethality of XRCC4- and Lig4-deficient mice, which can be rescued by p53 deficiency [45, 46]. Ku deficiency in mice also leads to increased p53-mediated death of newly generated neurons [49], but not as severely as that seen in XRCC4 or Lig4 deficiency and, correspondingly, Ku deficiency does not lead to embryonic lethality [50, 51]. Why Ku deficiency is less severe in this context is unknown but one hypothesis, based on the finding that Ku-deficiency can rescue the embryonic lethality of Lig4 deficiency [52, 53], is that Ku binding may block access of ends to alternative DSB repair pathways in XRCC4- or Lig4-deficient cells [1]. Deficiency for non-core C-NHEJ factors has a more variable and less severe effect for C-NHEJ events beyond the joining of CEs during V(D)J recombination. DNA-PKcs deficiency also leads to increased IR-sensitivity, genomic instability and reduced CSR but to a much lesser degree than observed for core C-NHEJ factor deficiencies [31, 32, 54–56]. Artemis deficiency also may variably impact these processes but to an even lesser extent than DNA-PKcs deficiency [32, 54, 57, 58]. Correspondingly, neither DNA-PKcs- nor Artemis-deficient mice have growth defects or neuronal defects [31, 50, 58].

C-NHEJ-deficient mice are not highly disposed to development of lymphoid or other cancers even though their developing and activated lymphocyte Ig and TCR loci translocate due to mis-joining of persistent RAG-initiated DSBs. Lack of tumor development in C-NHEJ-deficient mice is thought to be due to elimination of cells with persistent DSBs or oncogenic translocations via the p53-dependent G1/S checkpoint. Correspondingly, all tested C-NHEJ/p53 double-deficient mice, except XLF-deficient mice (see below), rapidly develop RAG-dependent pro-B lymphomas with IgH locus translocations that lead to *c-myc*

or, in the case of Artemis deficiency, *N-myc* oncogene amplification [45, 46, 59–62]. Core C-NHEJ-deficient mice that are also p53-deficient consistently develop medulloblastoma brain tumors, consistent with an important, but unknown, role of C-NHEJ in development of the nervous system [44, 48, 63, 64].

The XRCC4-like factor (XLF) [65, 66] has also been implicated in joining of DSBs, although its requirement for C-NHEJ appears variable and, in that regard, it is not required for robust developmental V(D)J recombination in mice [67, 68], due to a functional redundancy between XLF and various DSBR factors in C-NHEJ ([69–71]; discussed below) and a functional redundancy with DNA-PKcs in SE joining [32]. Correspondingly, germline deficiency for XLF in mice does not lead to any major impacts on survival or development, including that of lymphocytes. In the latter context, while there are modest effects on B and T cell development, these largely may be due to impacts on repair of DSBs other than those involved in V(D)J recombination [67, 68]. Also, consistent with functionally redundant factors that could compensate for XLF in end-joining, there is no obvious impact of XLF deficiency on neuronal development in mice. Due to the compensatory functions of XLF and the ATM-dependent DSBR, we will discuss XLF in more detail later in the review.

3. ATM-dependent DNA double-strand break response Proteins

The Ataxia telangiectasia (AT) mutated (ATM) protein kinase is a key upstream member of the ATM-dependent DSBR pathway [72]. ATM belongs to the phosphoinositide 3-kinase related protein kinase (PIKK) family that includes DNA-PKcs and Ataxia telangiectasia and Rad3-related protein (ATR) [73]. After DSB generation in G1, ATM activates several downstream factors including p53. Activation of p53 mediates the p53-dependent G1/S checkpoint to arrest cells with unrepaired DSBs to facilitate proper DSB repair or to cause apoptosis of cells with persistent DSBs [74–76]. The DSBR also participates directly in repair of DSBs, including those involved in V(D)J recombination [77, 78] and those involved in CSR [79]. Following activation via DSBs, ATM phosphorylates a set of proteins that includes histone H2AX, mediator of DNA damage checkpoint 1 (MDC1) and the p53-binding protein 1 (53BP1), which generate large foci in chromatin flanking DSBs [73]. In this regard, phosphorylated histone H2AX (“ γ -H2AX”) promotes recruitment of MDC1 [80], which contributes to the generation of a positive feedback loop that promotes spreading of phosphorylated H2AX over hundreds of kilobases (kb) within chromatin on either side of the DSB [81–84]. MDC1 also recruits ubiquitin ligases RNF8 and RNF168, the latter of which modifies H2A family histones (H2A and H2AX) to promote stable 53BP1 association within these foci [85–88]. Beyond potential roles in checkpoint signaling, formation of these ATM-dependent foci have been proposed to tether DSB ends for re-joining via C-NHEJ [89]. DSBR factors downstream of ATM also have been implicated in directing repair into C-NHEJ versus HR or A-EJ, for example by preventing end resection [90–94].

The human AT syndrome includes progressive ataxia, immunodeficiency, radio-sensitivity, genomic instability, increased Ig and TCR locus translocations in normal lymphocytes, and B and T cell lymphomas [95, 96]. The phenotype of ATM-deficient mice overlaps with that of AT patients and includes general cellular radio-sensitivity and genomic instability (as

determined cytogenetically), modest immunodeficiency, IgH CSR defects (30–50% of normal), and susceptibility to T cell lymphomas that all carry recurrent chromosomal translocations involving the TCR δ locus [97, 98]. Cytogenetic studies showed that most chromosomal aberrations in ATM-deficient cells, similar to those of C-NHEJ deficient cells [79, 99–101], occur in the form of chromosomal breaks and translocations, supporting the notion that ATM plays a most critical role during DSB repair in pre-replicative (e.g. G1) cells. While chromosomal translocations have not been well-characterized in immature ATM-deficient human T cell acute lymphocyte leukemias (“T-ALLS”), it seems likely that, as has recently been reported for human T-ALLs more generally, TCR δ locus translocations will also be a major feature of immature ATM-deficient human T-ALLs [102]. The CSR defects in ATM-deficient mice are associated with substantially impaired end-joining during CSR, which leads to unrepaired AID-initiated S region DSBs in activated B cells. These breaks progress at high frequency to chromosomal breaks and translocations observed by metaphase fluorescence *in situ* hybridization (FISH), supporting the notion that the DSB repair contributes to tethering S region DSBs for end-joining during CSR [79].

While there is clearly a substantial level of normal V(D)J recombination in AT patients and ATM-deficient mice, the frequent Ig or TCR locus translocations observed in normal T and B cells of AT patients and ATM-deficient mice were noted to be consistent with a V(D)J recombination joining defect [95]. With respect to roles of ATM in V(D)J recombination, studies that employed ATM-deficient mouse pro-B cell lines demonstrated that ATM deficiency or inhibition of ATM kinase activity leads to release of some CE from RAG-held V(D)J recombination post-cleavage complexes, leading to unrepaired RAG-generated DSBs that can progress to chromosomal breaks and translocations [78]. Such “free” CEs are also joined at increased frequency to cleaved SEs to generate hybrid or, potentially, open and shut joins instead of normal CJs [78]. Thus, ATM contributes to promoting proper C-NHEJ during V(D)J recombination by stabilizing RAG-mediated DSB post-cleavage complexes during V(D)J recombination [78]. Furthermore, ATM also has a role in SE joining that is revealed in the absence of DNA-PKcs [33, 103], that could reflect overlapping functions in phosphorylating common DNA repair substrates and/or in more direct roles in DNA end-tethering. With respect to downstream substrates, deficiencies for H2AX, MDC1 and 53BP1 DSB repair factors all also have clear-cut impacts on general C-NHEJ as best illustrated by effects on CSR; however, deficiencies for these factors have quite modest impacts on V(D)J recombination and lymphocyte development [81, 104, 105].

H2AX- and MDC1-deficient mice appear relatively normal, although their cells have increased IR-sensitivity and cytogenetic genomic instability [79, 81, 104]. Correspondingly, activated H2AX-deficient or MDC1-deficient B cells have a modest reduction in CSR associated with substantial levels of IgH locus chromosomal breaks, indicating roles for both in the end-joining phase of CSR. Cytogenetic studies revealed that H2AX and MDC1 likely function in DSB repair both during pre-replicative and post-replicative cell cycle phases [79, 81, 101], as evidenced by an increase in both chromosomal and chromatid breaks in H2AX-deficient or MDC1-deficient cells [79, 104, 106]. Neither H2AX deficiency nor MDC1 deficiency had any readily detectable impact on V(D)J recombination *in vivo* or on lymphocyte development. Yet, in a p53-deficient background, H2AX deficiency, or haplo-

insufficiency, predisposed to thymic lymphomas and to pro-B and B cell lymphomas [77]. Notably, H2AX/p53 double-deficient proB lymphomas harbored oncogenic IgH locus translocations with junctions that involve V(D)J recombination-associated DSBs, a finding that led to the proposal that H2AX may function in suppressing occasional generation of unrepaired DSBs during V(D)J recombination [77, 107]. In this regard, H2AX was subsequently shown to protect persistent RAG-initiated DSBs in C-NHEJ-deficient (e.g. Artemis- or Lig4-deficient) pro-B lines from undergoing aberrant resection [90] (also see below).

53BP1-deficient mice also appear relatively normal but again their cells have increased IR sensitivity [105, 108]. Yet, other than CSR-activated B cells, 53BP1-deficient cells have only modest, if any, cytogenetic instability. However, cytogenetic analyses of those cells that do show genomic instability indicate that 53BP1 deficiency mainly leads to chromosomal breaks consistent with a major 53BP1 role in genome stability maintenance occurring in pre-replicative cells as observed for ATM [70, 79, 99, 108, 109]. 53BP1-deficient mice have modestly reduced lymphocyte numbers and their differentiating T lymphocytes have very modest V(D)J recombination defects [105, 110, 111]. Strikingly, though, CSR is nearly abrogated in the absence of 53BP1 [105, 110]. In this context, CSR-activated 53BP1-deficient B cells have much greater levels of cytogenetic instability than other 53BP1-deficient cell types but nearly all of it is associated with AID-dependent IgH locus breaks and translocations.

The more dramatic CSR deficiency and associated IgH locus instability identified in 53BP1-deficient B cells compared to other DSB-deficient B cells indicates a more specialized role for 53BP1 in CSR, beyond that of the ATM-dependent DSBR [79]. Roles of 53BP1 that might contribute to CSR defects include protection of DSB ends from resection and potentially promoting their joining in the context of the DSBR [2, 79, 91–93, 105, 110, 111], although the overall mechanisms by which 53BP1 plays an especially crucial role in CSR compared to other DSBR factors remains to be elucidated. Finally, while 53BP1-deficient mice are not cancer prone, 53BP1-deficient mice that are also p53-deficient develop thymic or B cell lymphomas [109, 112], and 53BP1-deficient mice that have deregulated AID expression may also develop B cell lymphomas [113].

4. XLF is a C-NHEJ Factor but is not required for Normal V(D)J

Recombination in mice

The XRCC4-like factor (XLF, also known as Cernunnos, or Nhej1) was identified as a potential C-NHEJ factor through both cDNA complementation of cells derived from an IR-sensitive human immunodeficiency patient and through a yeast two-hybrid screen for XRCC4-interacting proteins [65, 66]. In humans, inactivating mutations of XLF result in an autosomal recessive disorder characterized by immunodeficiency that may become progressively more severe; but which, in general, is not as severe as the complete SCID phenotypes associated with human Artemis or DNA-PKcs deficiency [66, 114–116]. Similar to patients with hypomorphic mutations in Lig4, human XLF deficiency also is associated with microcephaly and radio-sensitivity [66, 116]. Moreover, XLF-deficient human fibroblasts that ectopically expressed RAG are substantially impaired for ability to undergo

V(D)J recombination in the context of episomal and intrachromosomal V(D)J recombination substrates, consistent with a role for XLF in C-NHEJ [66, 117].

Two independent XLF-deficient mouse lines were both relatively normal overall; but XLF-deficient MEFs and ES cells, similar to cells from XLF-deficient patients, had significant V(D)J recombination defects [67, 68]. Yet, XLF-deficient mice have only very mildly reduced peripheral T and B cell numbers. Moreover, distributions of developing B and T cells in the bone marrow and thymuses of XLF-deficient mice are comparable to those of WT counterparts [67, 68], in striking contrast to the block in lymphocyte differentiation at the progenitor stage of core C-NHEJ-deficient mice and DNA-PKcs- or Artemis-deficient mice [2, 25]. Abelson Murine Leukemia virus transformed pro-B cells (Abl pro-B cells) treated with the Abl kinase inhibitor Gleevec (STI571) undergo G1 cell cycle arrest, induce RAG expression, and carry out V(D)J recombination at the endogenous Ig κ light chain locus, as well as in the context of transient or chromosomally-integrated V(D)J recombination substrates [78]. XLF-deficient Abl pro-B lines performed V(D)J recombination on such substrates similarly to WT Abl pro-B lines, despite the fact that these lines were mildly IR-sensitive, suggesting a potential differential effect of XLF deficiency on DSB repair during V(D)J recombination versus more general DSB repair in these cells [32, 67, 70, 71].

Despite the lack of a V(D)J recombination defect in developing lymphocytes, other types of XLF-deficient cells show manifestations of C-NHEJ deficiency. Both XLF-deficient MEFs and XLF-deficient ES cells were IR-sensitive and impaired for joining V(D)J CEs and SEs within transient V(D)J recombination substrates [67, 68, 118], but in neither case was the impact as severe as is observed for XRCC4-deficient cells. Cytogenetic genomic instability is present in XLF-deficient MEFs and is primarily in the form of chromosomal breaks, implicating a role for XLF in repair of pre-replicative DSBs [32, 67, 70, 118]. Also consistent with an effect of XLF deficiency on general C-NHEJ, XLF-deficient mature B cells have reduced CSR coupled with increased unrepaired *IgH* chromosome breaks [67]. XLF/p53 double-deficient mice, unlike other C-NHEJ/p53 double-deficient mice, rarely die of pro-B cell lymphomas; but rather die of T cell lymphomas characteristic of p53 deficiency [67]. As C-NHEJ/p53 double-deficient pro-B cell lymphomas routinely contain oncogenic translocations involving RAG-generated *IgH* locus DSBs, the rarity of this type of tumor in XLF/p53 double-deficient mice is consistent with normal V(D)J recombination in developing XLF-deficient lymphocytes. Notably, however, many XLF/p53 double-deficient mice also develop medulloblastomas (MBs), as seen in other C-NHEJ/p53 double-deficient mice [17, 119, 120]. The occurrence of recurrent MBs in XLF/p53 double-deficient mice is consistent with a general C-NHEJ defect in MB progenitors and warrants deeper investigation into underlying mechanisms. As XLF clearly functions as a C-NHEJ factor in a broad variety of cell types, the relatively normal V(D)J recombination in XLF-deficient progenitor lymphocytes and Abl pro-B cell lines coupled with the normal lymphocyte development in XLF-deficient mice [67, 68], suggested the existence of factors that compensate for XLF function in C-NHEJ, specifically in the context of V(D)J recombination in developing lymphocytes [67].

Most current models for XLF function derive from biochemical and structural assays, which suggest that XLF largely works in concert with the structurally similar XRCC4 factor. Both XLF and XRCC4 contain a globular head domain, an alpha-helical stalk domain, and an unstructured C-terminal domain [65, 121]. In this regard, XLF forms heterodimers with XRCC4 through interactions of their respective globular head domains [65, 121]. Known functions of XRCC4 include stabilization of Lig4 through direct interaction [122] and stimulation of Lig4 activity [123]. XLF/XRCC4 heterodimers form protein filaments [121, 124–127] that tether DNA ends *in vitro* and which have been speculated to enhance XRCC4-dependent recruitment of Lig4 to DSBs [124, 126]. In biochemical assays, XLF also enhances efficiency of the XRCC4/Lig4 complex to ligate linearized plasmids *in vitro* [128]. Notably, non-lymphoid cells that express mutant forms of XRCC4 that are unable to interact with XLF carry out joining of SEs, but not CE, in the context of extra-chromosomal V(D)J recombination substrates [129], suggesting potential functional compensation for XLF-XRCC4 end-bridging by RAG or other factors in the post-cleavage synaptic complex for joining of SEs [121, 129, 130].

The findings that XLF is not required for V(D)J recombination in normal developing lymphocytes but is required for ectopic V(D)J recombination in non-lymphoid cells led to the hypothesis that other factors may functionally compensate for XLF to promote V(D)J recombination in developing lymphocytes [67]. As mentioned above, one such factor might be RAG, which holds CE and SE in a post-cleavage synaptic complex and directs C-NHEJ-mediated joining of two CE and two SEs, respectively, to each other [131, 132]. In this context, SEs are held more tightly than CE in the RAG post-cleavage synaptic complex [133]; which in the context of proposed XLF synapsis functions could explain the lack of impact on SE joining versus CE joining when XRCC4-XLF interaction is disrupted [129]. If RAG does function redundantly with XLF during V(D)J recombination in developing lymphocytes, but not in non-lymphoid cells, it is conceivable that normal physiological V(D)J recombination may have evolved to optimize ability of RAG, for example via post-translational modifications, to hold CE and/or SE in post-cleavage synaptic complexes for proper end-joining and/or to contribute to C-NHEJ factor recruitment [12, 131, 132, 134]. However, additional studies, described below, demonstrated that factors other than RAG provide functional redundancy with XLF in V(D)J recombination and C-NHEJ more generally.

5. XLF has functional redundancy with ATM

The ATM-dependent DSBR is activated in response to endogenous RAG-initiated DSBs at antigen receptor loci [135, 136], and deficiencies for ATM or several ATM downstream factors (H2AX and 53BP1) impair end-joining during V(D)J recombination, with impacts ranging from moderate for ATM [78] to very modest for H2AX [79, 104, 106]. Such studies led to the proposal that the ATM-dependent DSBR, beyond activating checkpoints, may contribute to C-NHEJ through end-tethering during V(D)J recombination, CSR, and more generally [1, 2, 79, 89, 137]. Indeed, ATM was found to stabilize RAG-mediated V(D)J breaks in the context of the post-cleavage complex [78]. Together, these findings led to the evaluation of ATM and downstream DSBR factors as candidates for having functional redundancy with XLF, potentially through roles in end-tethering. Correspondingly,

XLF/ATM double-deficient mice were observed to have a severe block in B and T cell development at the progenitor stage when V(D)J recombination occurs, very reminiscent of the SCID phenotype of C-NHEJ-deficient mice [71]. In the XLF/ATM double-deficient background, the B cell developmental block was substantially rescued by introduction of germline alleles containing pre-assembled IgH and IgL variable region exons ("HL alleles"), consistent with impairment resulting largely from a V(D)J recombination defect [71]. Correspondingly, XLF/ATM double-deficient Abl pro-B cells arrested in G1 to activate V(D)J recombination accumulated unrepaired breaks in the endogenous Ig κ locus [71], and were severely impaired in ability to join CEs and SEs of RAG-initiated DSBs within chromosomally integrated V(D)J recombination substrates. Thus, combined deficiency for ATM and XLF essentially abrogates chromosomal V(D)J recombination in developing lymphocytes (Figure 2).

The studies of developing lymphocytes or pro-B cell lines clearly demonstrate that XLF-deficient B lineage cells require ATM and ATM-deficient B cells require XLF to perform C-NHEJ during V(D)J recombination [71]. XLF/ATM double-deficient mouse fibroblasts have substantially increased cytogenetic instability as compared to fibroblasts deficient for either factor alone (Oksenych and Alt, unpublished), which among other possibilities, suggests that ATM and XLF may have functional redundancy in C-NHEJ more generally. Indeed, studies of CSR in the XLF/ATM double-deficient background strongly support this notion. Thus, CSR to IgG1 in mature XLF/ATM double-deficient B cells generated in a background containing HL alleles was reduced to the residual levels observed in core C-NHEJ-deficient cells (Figure 2). As residual CSR in C-NHEJ-deficient B cells is known to occur via A-EJ, these findings also suggested that the observed functional redundancy for ATM and XLF primarily occurs in the context of C-NHEJ and not for A-EJ [71].

Assays for V(D)J recombination within extra-chromosomal substrates introduced into XLF/ATM double-deficient Abl pro-B cells revealed that both CE and SE joining occurred at levels similar to those of WT Abl pro-B cells, in striking contrast to the essentially complete block in chromosomal V(D)J recombination in XLF/ATM double-deficient lines [71]. This finding suggested that XLF and ATM functional redundancy may be more specific to chromosomal V(D)J recombination in the context of chromatin [71]. In this regard, inhibition of ATM kinase activity in XLF-deficient Abl pro-B cells also led to a block in joining of RAG-cleaved CEs and SEs within chromosomally integrated V(D)J recombination substrates, suggesting that XLF-redundant functions of ATM in chromosomal V(D)J recombination joining may be mediated, at least in part, by downstream ATM substrates in the context of chromatin [71]. These findings led to additional studies that implicated H2AX and 53BP1 as factors that have functional redundancy with XLF in C-NHEJ during V(D)J recombination.

6. XLF has redundant functions with ATM substrates H2AX and 53BP1

Combined deficiency for XLF and histone H2AX results in early embryonic lethality. The cause of such embryonic lethality remains to be determined. One proposed model is that embryonic lethality may be caused by XLF and H2AX functional redundancy in DNA repair processes beyond those shared with ATM alone. Such functions, for example, might be

related to potential ATM-independent post-replicative DNA repair roles for H2AX as suggested by the increased levels of chromatid breaks in H2AX-deficient versus ATM-deficient cells [71, 79, 81, 101]. In this context, H2AX can be activated independently of ATM by ATR, DNA-PKcs [71, 138–141] and, conceivably, by other kinases. If this model is correct, one might also expect MDC1 deficiency, which also leads to defects in replicative or post-replicative DSB repair defects, to lead to embryonic lethality when combined with XLF deficiency. Another, non-mutually exclusive model, in analogy to p53 rescue of embryonic lethality of Lig4- or XRCC4-deficient mice [46], would be that checkpoint defects associated with ATM deficiency, but not H2AX deficiency, rescue potential embryonic lethal DNA repair defects of XLF/ATM deficient mice [71]. If correct, p53 deficiency might rescue the embryonic lethality of XLF/H2AX deficiency. However, it is also notable that XLF/H2AX embryonic lethality occurs much earlier than the late embryonic lethality of Lig4 or XRCC4 deficiency, both of which are associated with dramatic neuronal apoptosis [45–47, 71].

It was not possible to assess potential defects in lymphocyte development or DNA repair in the context of XLF/H2AX double-deficient mice due to early embryonic lethality. However, conditional inactivation of H2AX in XLF-deficient Abl pro-B lines had no impact on cellular viability. In these lines, combined H2AX and XLF deficiency also results in severely impaired joining of RAG-generated CEs and SEs; albeit not quite to the same extent as seen with dual ATM- and XLF-deficiency [71] (Figure 2). However, while in XLF/ATM double-deficient Abl pro-B lines, un-joined CEs and SEs appeared largely intact, in XLF/H2AX double-deficient Abl pro-B lines most un-joined CEs and SEs were highly resected, consistent with findings that H2AX protects such ends from resection in C-NHEJ-deficient backgrounds [90]. At first glance, this differential impact of ATM and H2AX deficiency on the fate of un-joined CEs and SEs in an XLF-deficient background might not seem consistent with such H2AX functions lying downstream of ATM. However, ATM deficiency has a dual impact on resection of un-joined CEs and SEs during V(D)J recombination, both protecting these ends from resection via generation of phosphorylated γ H2AX and also promoting their resection by activating CtIP [90]. Thus, the defect in CE and SE joining *per se* in the combined absence of H2AX and XLF may reflect H2AX functioning downstream of ATM activation at a DSB, both to promote joining, for example via an end-tethering function, and also to specifically prevent end resection by ATM-activated CtIP. Correspondingly, inhibition of ATM kinase activity in XLF/H2AX double-deficient Abl pro-B cell lines substantially restored accumulation of un-joined CEs and SEs that were not markedly resected.

XLF and 53BP1 double-deficient mice are live born, but compared to XLF-deficient or 53BP1-deficient mice, are growth retarded, their fibroblasts have increased cytogenetic genomic instability manifested primarily as chromosome breaks, and they are more prone to thymic lymphoma [69, 70]. Moreover, like XLF/ATM double-deficient mice, XLF/53BP1 double-deficient mice have a SCID phenotype with lymphocyte development essentially blocked at the progenitor B and T cell stages, consistent with impaired V(D)J recombination [69, 70]. Indeed, analyses of XLF/53BP1 double-deficient Abl pro-B cell lines revealed a combined impact on ability to join RAG-generated CEs and SEs during V(D)J

recombination that was strikingly similar to the combined impact of dual XLF/H2AX deficiency (Figure 2), including severely impaired joining of both CEs and SEs and dramatically increased resection of the un-joined CEs and SEs that was rescued by treatment of cells with an ATM inhibitor [69, 70]. The end protection role of 53BP1 is consistent with such roles in other contexts including CSR [91–94]. While the V(D)J recombination defects of XLF/53BP1 double-deficient Abl pro-B lines was substantial, as seen with dual XLF/H2AX deficiency, some residual joining remained [69, 70]. In this context, the essentially complete SCID phenotype of XLF/53BP1 double-deficient mice may reflect both greatly impaired V(D)J recombination, as well as an impact on developing or more mature lymphocytes due to effects on general C-NHEJ as suggested by the increased genomic instability of XLF/53BP1 double-deficient fibroblasts.

Overall, the impact of H2AX or 53BP1 deficiency on V(D)J recombination and C-NHEJ in an XLF-deficient background is consistent with both DSB proteins having critical functions in both end-joining *per se* (e.g. by end-tethering roles) and end protection from aberrant resection in the context of the ATM-dependent DSB. As 53BP1 may also have functions at DSBs independent of ATM, as it likely does during CSR, a functional redundancy with XLF in this context cannot be ruled out. XLF, on its own, does not appear to have a major role in protecting un-joined RAG-initiated CEs and SEs from resection. Thus, these ends are highly resected in Lig4/H2AX double-deficient or Artemis/H2AX double-deficient Abl pro-B cells [90]. Likewise, in Artemis-, DNA-PKcs-, or XRCC4-deficient Abl pro-B cells that are also XLF-deficient, persistent, unrepaired CEs generated during attempted V(D)J recombination do not undergo increased resection [32].

7. XLF and DNA-PKcs have Functional Overlaps in V(D)J recombination and C-NHEJ

DNA-PKcs and ATM are related PIKKs with shared substrates [140], are functionally redundant with respect to SE joining during V(D)J recombination [33, 103], and have both been suggested to potentially play a role in tethering DSB ends during C-NHEJ [39–41, 78]. Correspondingly, DNA-PKcs also appears to have functional overlap with XLF in C-NHEJ. Thus, XLF/DNA-PKcs double-deficient fibroblasts have increased levels of chromosomal breaks compared to that of DNA-PKcs- or XLF-deficient fibroblasts [32]. Moreover, combined deficiency for XLF and DNA-PKcs abrogates joining of RAG-initiated SEs within chromosomally integrated V(D)J recombination substrates in Abl pro-B cells [32]. Given that XLF/Artemis double-deficient Abl pro-B cells did not display such an impact on SE joining, the dramatically impaired SE joining in XLF/DNA-PKcs double-deficient Abl pro-B cells involves Artemis-independent DNA-PKcs functions. In this context, XLF/DNA-PKcs double-deficient mice are live born but die shortly after birth for unknown reasons; whereas XLF/Artemis double-deficient mice do not exhibit early post-natal lethality, consistent with XLF/DNA-PKcs functional redundancy separate from DNA-PKcs function in Artemis activation [32]. Finally, the apparent DNA-PKcs redundant function with XLF is mediated by its kinase activity, as DNA-PKcs kinase inhibition in XLF-deficient Abl pro-B cells or mature B cells abrogates SE joining and reduces CSR levels, respectively [32]. The

latter finding also is consistent with a broader functional redundancy between XLF and DNA-PKcs in C-NHEJ in general.

8. Perspectives

The finding that the ATM-dependent DNA damage response results in the formation of large foci in chromatin surrounding DSBs [142, 143] raised the possibility of a direct role for the DSBR in DSB repair, and, in particular, in C-NHEJ. Likewise, the discovery that XLF is required for protection from ionizing radiation and also for joining of RAG-initiated DSBs in patient fibroblasts implicated this XRCC4-related factor as a potential C-NHEJ component [66, 144]. A confounding issue, however, in considering potential roles of ATM-dependent DSBR factors and the XLF protein as C-NHEJ factors was the relative dispensability of these factors for C-NHEJ during V(D)J recombination. However, the finding that XLF shares redundant functions with ATM and several of its downstream DSBR factors with respect to C-NHEJ during V(D)J recombination in developing lymphocytes greatly clarified the critical roles of XLF and the DSBR in this process. Thus, in the absence of XLF, developing B and T lymphocytes are totally reliant on ATM and ATM downstream factors to carry out normal chromosomal V(D)J recombination; likewise, in the absence of ATM or downstream factors, developing B and T cells require XLF for C-NHEJ during V(D)J recombination. Indeed, while XLF deficiency has little impact on lymphocyte development, and ATM deficiency has only a modest impact, combined ATM and XLF deficiency results in a SCID phenotype reminiscent of that observed in the context of C-NHEJ deficiency [71]. A further surprising recent finding is the additional functional redundancy between XLF and the DNA-PKcs in C-NHEJ [32].

The ongoing challenge is to elucidate the nature of the redundant functions of XLF and DSBR factors (and DNA-PKcs) and whether they have overlapping roles in the same general function (e.g. end-tethering) (Figure 3) or whether they represent roles in different processes that provide different functions (e.g. end-tethering versus C-NHEJ factor recruitment) that are compensatory for each other in C-NHEJ [71]. A potential example of compensatory but different functions might include DNA end-tethering and C-NHEJ factor recruitment (or activation) (Figure 4). Thus, efficient tethering of DSBs may keep them together long enough for ligation even at reduced repair efficiency due to impaired C-NHEJ factor recruitment; conversely, efficient C-NHEJ recruitment might rapidly repair ends before they separate due to reduced tethering activity (Figure 4). With respect to such potential roles in C-NHEJ, DNA-PKcs and XLF have been directly implicated in DNA end-tethering [39–41, 121, 124–127], while ATM and its downstream DSBR factors have been suggested to play such roles either directly or indirectly [78, 79, 89, 105, 111]. With respect to C-NHEJ factor recruitment or activation, XLF was originally described as a part of the XLF/XRCC4/Lig4 complex with potential roles in the ligation phase of the reaction [65, 128, 145], and XLF has been found to stimulate Lig4 activity [128, 145].

It is notable that single deficiencies for XLF or individual DSBR factors generally have a less pronounced effect on V(D)J recombination than on other forms of C-NHEJ-mediated DSB repair including CSR. In theory, the lower V(D)J recombination impact might reflect a contribution by RAG in holding CE and SE in synaptic complexes and/or promoting their

joining by C-NHEJ [12, 131, 132]. In this context, ATM or DNA-PKcs phosphorylation of RAG is not required for its function in normal cells [146]; but whether such an activity could contribute to the impact of ATM or DNA-PKcs deficiency on V(D)J recombination in the absence of XLF remains to be tested. Finally, functional redundancies between XLF and DSB factors or RAG might also contribute to the phenotypic diversity of deficiencies for these factors in human patients or between humans and mice. For example, the marked variation in degree of lymphopenia observed in XLF-deficient patients [66, 116] might reflect variations in the expression of XLF compensatory proteins. Conversely, the impact of ATM deficiency in humans can vary among patients [95, 147, 148], and ATM-deficient mice do not exhibit the overt neurological defects observed in humans [95, 98]. Potentially, such variations could also reflect, at least in part, varying degrees of XLF compensation for ATM function in these different settings.

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Bullet points

- XLF is a DNA repair protein that functions in C-NHEJ.
- XLF has functional redundancy with multiple DSB response factors including ATM.
- XLF and the DSB response have redundant roles in B and T cell development.
- XLF and several DSB response factors have redundant roles in general DSB repair.
- XLF and certain DSB response factors have redundant roles in mouse development.

Figure 1. V(D)J recombination in developing lymphocytes requires the RAG endonuclease and C-NHEJ factors

This Figure depicts an example of a V(D)J recombination event occurring between a V gene segment and a rearranged DJ gene segment to form a complete V-D-J exon.

(A) The RAG1/2 endonuclease (green ovals) is recruited to recombination signal (RS) sequences flanking a “V” gene segment and a “DJ” gene segment. The white triangle represents a 23-RSS, and the black triangle represents a 12-RSS.

(B) After DSB induction, RAG holds hairpin-sealed coding ends and blunt signal ends in a post-cleavage synaptic complex.

(C) The Ku70/Ku80 heterodimer (denoted by the light blue semicircle and dark blue semicircle, respectively) recognizes and binds DNA ends. Hairpin-sealed coding ends require DNA-PKcs (light blue oval) and the nuclease Artemis (red circle) to open and process the hairpins before ligation.

(D) DNA ligase 4 (Lig4, yellow oval) and XRCC4 (orange oval) are recruited to the DNA repair complex in a Ku-dependent manner. XLF (orange circle) is also recruited to the DNA repair complex by Ku.

(E) The XRCC4/Lig4 complex ligates signal ends and coding ends, resulting in signal joins (left) and coding joins (right), respectively.

Figure 2. XLF has functional redundancy with ATM and ATM substrates (H2AX, 53BP1) during C-NHEJ

(A) Factors of the DSBR pathway (ATM, γ H2AX, 53BP1; pink oval) accumulate around a DSB. Ku70/Ku80 (light blue and dark blue semicircles) bind DNA ends. DNA-PKcs (light blue oval), XRCC4/Lig4 (orange and yellow ovals) and XLF (orange circle) are recruited to the C-NHEJ DNA repair complex by Ku.

(B) and (C) In the absence of ATM (or H2AX, or 53BP1), C-NHEJ is functional. In the absence of XLF, C-NHEJ is also largely functional.

(D) In cells with combined deficiency for XLF and ATM, C-NHEJ is nearly abrogated. In cells with combined deficiency for XLF and H2AX, or XLF and 53BP1, C-NHEJ is dramatically reduced. It remains unclear which stage of C-NHEJ is defective in the combined absence of XLF and ATM or its substrates (as denoted by the question marks).

Figure 3. XLF and ATM (and DSB factors) have redundant functions during C-NHEJ
 (A) Both ATM (pink oval) and XLF (orange circle) may stimulate Lig4 (yellow oval) activity, and/or stabilize the XRCC4/Lig4 (orange and yellow ovals) complex at a DSB.
 (B) ATM and its downstream substrates, combined with XLF/XRCC4 filaments, may tether DNA ends in close proximity before ligation.

Figure 4. XLF and ATM (and DSBR factors) have complementary functions during C-NHEJ
 (A) ATM (pink oval) may function to hold DNA ends, while XLF (orange circle) stimulates XRCC4/Lig4 (orange and yellow ovals) activity.
 (B) ATM may stimulate XRCC4/Lig4, while XLF/XRCC4 filaments hold DNA ends.