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The recent advances in non-homologous end-joining through the lens of lymphocyte development

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Abstract

Lymphocyte development requires ordered assembly and subsequent modifications of the antigen receptor genes through V(D)J recombination and Immunoglobulin class switch recombination (CSR), respectively. While the programmed DNA cleavage events are initiated by lymphocyte-specific factors, the resulting DNA double-strand break (DSB) intermediates activate the ATM kinase-mediated DNA damage response (DDR) and rely on the ubiquitously expressed classical non-homologous end-joining (cNHEJ) pathway including the DNA-dependent protein kinase (DNA-PK), and, in the case of CSR, also the alternative end-joining (Alt-EJ) pathway, for repair. Correspondingly, patients and animal models with cNHEJ or DDR defects develop distinct types of immunodeficiency reflecting their specific DNA repair deficiency. The unique end-structure, sequence context, and cell cycle regulation of V(D)J recombination and CSR also provide a valuable platform to study the mechanisms of, and the interplay between, cNHEJ and DDR. Here, we compare and contrast the genetic consequences of DNA repair defects in V(D)J recombination and CSR with a focus on the newly discovered cNHEJ factors and the kinase-dependent structural roles of ATM and DNA-PK in animal models. Throughout, we try to highlight the pending questions and emerging differences that will extend our understanding of cNHEJ and DDR in the context of primary immunodeficiency and lymphoid malignancies.

Keywords

Non-homologous end-joining; ATM; DNA-PK; V(D)J recombination; Class Switch Recombination

1. Introduction

Mammalian cells have two major DNA double-strand break (DSB) repair pathways – the classical non-homologous end-joining (cNHEJ) pathway that directly ligates two DNA ends together, and the homologous recombination (HR) pathway that repairs a DSB using the sister chromatid or a homologous chromosome as the template. In recent years, the alternative end-joining (Alt-EJ) pathway has been used to describe end-ligation in cells lacking core cNHEJ factors (*e.g.*, KU, LIG4, XRCC4). Alt-EJ preferentially uses microhomology (MH) at the junctions, is mediated by single-strand DNA ligases, LIG1 and LIG3 with the help of POLQ, and partially overlaps with the microhomology (MH)-mediated end-joining (MMEJ) pathway originally described in yeast[1–4]. Since the cNHEJ pathway can also generate joining products with short MH (n<=4nt), we chose to use the term Alt-EJ here to describe end-ligations without core cNHEJ components regardless the exact number of MH usage. In addition to the specific repair pathways that work on the DNA, DSBs also activate ATM kinase and a cascade of post-translational modifications, collectively referred to as the DNA damage response (DDR), which promotes precise and efficient DSB repair in part by modifying the chromatin environments. There are excellent reviews on the detailed molecular mechanism of cNHEJ[5,6], Alt-EJ[1–4], and DDR[7,8], here, we focus on the roles of DDR and cNHEJ on lymphocyte-specific gene rearrangements.

The mammalian adaptive immune system is renowned for its specificity, diversity, and memory. This is achieved at the DNA level through three programmed gene rearrangement events– V(D)J recombination, Immunoglobulin class switch recombination (CSR), and somatic hypermutation (SMH). SHM introduces mutations in the variable regions of Immunoglobulin (Ig) genes in B lymphocytes via the base excision repair and mismatch repair pathways without evidence for DNA DSBs[9]. Here we focus on the two events involving DNA DSB intermediates - V(D)J recombination and CSR. V(D)J recombination occurs in progenitor B and T lymphocytes, where the variable region exons encoding the antigen-binding domains are assembled from individual Variable (V), Diversity (D) and Joining (J) gene segments through a cut and paste mechanism (Fig. 1). V(D)J recombination occurs in all T cell receptor (TCR, α/δ, β, γ) and Ig (h, κ, λ) gene loci[10], including the Ig Heavy Chain (IgH), depicted as an example in Figure 1. CSR occurs in naïve B cells upon antigen exposure and T cell contact, and replaces the initially expressed IgM constant region (Cμ) with another constant region (*e.g.*, Cγ1 for IgG1), to generate an antibody with a different isotype, thus conferring a different effector function (Fig. 1). There are excellent reviews on the initiation and regulation of V(D)J recombination[11–13] and CSR[9,14,15], here we focus on the DSB repair phase. The DSB intermediates generated during V(D)J recombination and CSR activate the ATM kinase-mediated DDR and use the ubiquitous cNHEJ pathway, and, in the case of CSR, also the Alt-EJ pathway for completion[16]. Both V(D)J recombination and CSR are initiated in the G1 phase of the cell cycle when

homologous templates are not readily available, explaining the lack of direct contribution from the HR pathway. That is been said, several HR factors contribute to lymphocyte development by supporting efficient proliferation and clonal expansion[17,18]. V(D)J recombination is completed within the G1 phase of the cell cycle and involves hairpin end intermediates[19]. In contrast, CSR occurs in proliferating cells and involves DSBs at highly repetitive and GC-rich switch regions[15]. These and other features explain the unique immunodeficient phenotypes associated with different DNA repair defects and also provide a valuable tool to understand the mechanisms of cNHEJ and DDR in general. Here, we compare and contrast the physiological roles of different cNHEJ and DDR factors during V(D)J recombination and CSR with an emphasis on newly discovered cNHEJ factors, the kinase-dependent structural functions of DNA-dependent protein kinase (DNA-PK), and the interplay between ATM-mediated DDR and cNHEJ (Table 1). Whenever possible, we try to highlight the challenges and new opportunities in understanding DNA repair during lymphocyte development using animal models and their implications in human diseases, especially immunodeficiency and lymphoid malignancies (Table 1). The clinical immunodeficiency associated with defects in cNHEJ and DDR are reviewed elsewhere[20,21].

2. DNA DSB repair during V(D)J Recombination

Recombination activating gene (RAG) - RAG1 and RAG2 - initiate V(D)J recombination in progenitor lymphocytes by recognizing a pair of compatible recombination signal sequences (RSSs) and introducing DSBs between the RSSs and the participating V, D, or J gene segments[11] (Fig. 1). RAG form a Y-shape hetero-tetramer to synapse the involving V, D or J segments together and coordinately cleavage the two RSSs to generates two types of ends – a pair of covalently sealed hairpinned coding ends (CEs) and a pair of blunt and 5’ phosphorylated signal ends (SEs) (Fig. 2A). The top of the Y formed by RAG2 tilts toward one side to accommodate the two RSSs with different sizes of spacers (12bp and 23bp, respectively), which explain the 12–23 rule for RAG mediated recombination (Fig. 2B)[22,23]. After cleavage, the CEs are released and RAG holds the two SEs in a hairpin-forming complex (Fig. 2B) and promotes the ligation between the two SEs to generate a signal join. The two CEs undergo hairpin-opening and end-processing via the cNHEJ pathway before they are ligated to form a coding join (CJ) that eventually becomes part of the variable region exons (Fig. 2C). RAG expression is restricted to G1 arrested progenitor lymphocytes[24] and RAG dictates the orientation-specific repair outcome of V(D)J recombination, explaining the exclusive dependence of V(D)J recombination on cNHEJ. RAG cleavage only occurs in transcriptionally active and epigenetically poised V, D, and J gene segments[10,25], leading to the cell type (B vs T) and developmental stage (pro- vs pre-) specificity of V(D)J recombination.

2.1. The role of cNHEJ in V(D)J recombination

The cNHEJ pathway is exclusively required for V(D)J recombination[26,27] and entails both an evolutionarily conserved end-ligation component and a largely vertebrate-specific end-processing component (Fig. 2D). Although putative orthologs of DNA-PKcs has been identified in other non-vertebrate species based on sequence homology, the biological function of DNA-PKcs has not been characterized. During V(D)J recombination, KU70

(gene name XRCC6) and KU86 (Ku80 in mouse, gene name XRCC5) form the KU heterodimer, which binds to hairpin CEs and blunt SEs and initiates cNHEJ. For end-ligation, KU recruits and stabilizes the ligation complex formed by XLF (gene name NHEJ1), XRCC4, and LIG4 to ligate the blunt SEs directly and precisely, forming a SJ[28–30]. For end-processing, KU, especially the C-terminus of KU80[31], recruits and activates the catalytic subunit of DNA-PK (DNA-PKcs, gene name PRDKC), which activates Artemis endonuclease to open the CE hairpins[32,33], thereby allowing the CEs to be ligated to form a CJ. Hairpin opening outside the apex introduces palindromic insertions (P elements)[32–36] and terminal deoxynucleotidyl transferase (TDT) also adds non-templated insertions (N nucleotides)[37,38] to further increase the diversity of the variable region exons. KU associated DNA polymerases (λ and μ)[39,40] and WRN helicase[41,42] further contribute to the V(D)J recombination. Correspondingly, mouse models deficient in end-ligation caused by loss of Ku70, Ku80, Lig4, or Xrcc4 - display severe neuronal apoptosis and accumulate both CEs and SEs[43–47] (Table 1). In the case of Lig4, or Xrcc4, the neuronal apoptosis is accompanied by embryonic lethality that can be rescued by the co-deletion of KU[48], which normally limits end-resection[49] and Alt-EJ mediated repair. While Xlf-null mice do not develop overt neuronal apoptosis, patients with XLF/Cernunnos deficiency show microcephaly, consistent with a model in which end-ligation prevents neuron apoptosis[30,50]. Meanwhile, mice with complete loss of the largely vertebrate-specific end-processing factors DNA-PKcs or Artemis have no neuronal apoptosis, carry isolated immunodeficiency, and form SJs efficiently but with reduced fidelity[51–53] (Table 1). Similarly, patients with complete loss of Artemis[33] or DNA-PKcs mutation that only affects the hairpin opening and Artemis activation[54] did not develop marked microcephaly. Notably, mouse models expressing a kinase-dead DNA-PKcs that blocks DNA end-ligation also have severe neuronal apoptosis and late embryonic lethality[55], like *Lig4*$^{-/-}$ or *Xrcc4*$^{-/-}$ mice, highlight the importance of end-ligation in neurological development. In this regar, a patient with substantially decreased expression of catalytically inactive DNA-PKcs protein also suffers profound neurological abnormalities and show sign of end-ligation defects[56]. Together, the data supports an important role of cNHEJ mediated end-ligation in post-mitotic neurons *in vivo*. The cNHEJ factors discussed in this section contibutes to V(D)J recombination by directly working on the DNA itself (in contrast to chromatin) and have also been extensively characterized using plasmid-based substrates (Table 1).

2.2. The role of DNA-PK and its kinase activity in cNHEJ and V(D)J recombination

The KU heterodimer and DNA-PKcs together form the DNA-PK holoenzyme[57] that is related to the ATM kinase[58]. V(D)J recombination has been a valuable system to study the function of DNA-PK. DNA-PKcs is the best-characterized substrate of DNA-PK. To understand its regulation *in vivo*, we generated a mouse model expressing kinase-dead (KD) DNA-PKcs (D3922A, *DNA-PKcs*^{KD})[55,59]. In contrast to the normal development of DNA-PKcs null mice[51,52,60], *DNA-PKcs*$^{KD/KD}$ mice die *in utero* with severe neuronal apoptosis like in *Lig4*- or *Xrcc4*- deficient mice[55], and accumulate both CEs and SEs, indicative of end-ligation defects[55] (Table 1). Nevertheless, the deletion of KU or the Ku80 C-terminal domain that recruits DNA-PKcs to the DNA ends rescues the embryonic lethality and SJ formation defects in *DNA-PKcs*$^{KD/KD}$ mice[55], indicating that, once loaded to DNA ends, DNA-PKcs requires its kinase activity to “license” end-ligation. Together with the

observation that purified DNA-PKcs blocks DNA ligation by a T4 DNA ligase in the absence of hydrolyzable ATP[61], these findings support a cap function of DNA-PKcs that is regulated by its own kinase activity. A recent study using ectopically expressed DNA-PKcs KD protein in human cells did not detect additional end-ligation defects

ATP hydrolysis is also essential for Artemis and DNA-PK mediated hairpin opening *in vitro*[32]. To identify the phosphorylation targets of DNA-PKcs, alanine substitutions were introduced to either Artemis or DNA-PKcs, the results suggest that DNA-PKcs phosphorylation at the T2609 cluster facilitates Artemis activation[62]. Denature gel electrophoresis shows that the hair pinned CEs are fully opened in *DNA-PKcs*$^{KD/KD}$ cells and that hairpin opening occurs efficiently in *DNA-PKcs*$^{KD/KD}$ cells and can be blocked by ATM kinase inhibitor[55], suggesting that Artemis activation requires DNA-PKcs protein and the kinase activity from either DNA-PK or ATM. A patient carrying R3062L mutation in DNA-PKcs retains full kinase activity, yet loss the ability to activate Artemis[20,54,56], also consistent with a structural rather kinase role of DNA-PKcs in Artemis activation. Given the strict requirement for DNA-PK's kinase activity for end-ligation and the redundancy between ATM and DNA-PK activity for end-processing, we initially speculated that these differential requirements might reflect specific roles for DNA-PKcs autophosphorylation vs DNA-PKcs transphosphorylation by ATM.

Human DNA-PKcs has two well-characterized phosphorylation clusters[63]. Upon DSBs (*e.g.*, radiation), the S2056 cluster is mainly inter-molecularly auto-phosphorylated by DNA-PK[64–66] and the T2609 cluster is phosphorylated by ATM[64,67–69] and DNA-PK[65,70]. The relative contribution of ATM vs DNA-PK to the T2609 cluster phosphorylation varies by relative abundance and activities of ATM vs DNA-PK. Upon UV irradiation, ATR kinase can also phosphorylate DNA-PKcs at the T2609 cluster[71]. Although not essential for cNHEJ in human cell extracts *in vitro*[72], expression of human DNA-PKcs with an alanine substitution at the S2056 and/or T2609 cluster in DNA-PKcs-deficient CHO cells fails to restore IR resistance[63,73–75] and Artemis mediated end-processing[62,76]. Moreover, episomal analyses of the DNA-PKcs with alanine substitutions at T2609 or S2056 clusters show reduced end-ligation that is comparable to the loss of DNA-PKcs[65,73] and loss of both S2056 and T2609 cluster phosphorylation has a synergistic effect on end-ligation and IR sensitivity[64,65,73]. Yet, despite mild IR sensitivity, mouse models with knock-in alanine substitutions at either the S2056 cluster[77] or the T2609 cluster[78] support chromosomal V(D)J recombination (Table 1)[77–80]. Given DNA-PKcs is not essential for SJ formation during chromosomal V(D)J recombination or end-joining, the results support a model in which DNA-PKcs phosphorylation at the T2609 and S2056 cluster promotes end-ligation, but are not essential for either end-processing and end-ligation[72].

That is being said, there is several notably difference among results acquired from different experimental systems. While additional experiments are necessary to resolve the difference, we offer some thoughts for consideration. First, chromosomal V(D)J recombination vs episomal reporters. In general, loss of DNA-PKcs and its phosphorylation causes more severe defects on plasmid-based assays than on chromosomal substrates or *in vivo*. One possibility is that the chromatin and chromatin bounded DNA damage response factors promote end-ligation and mask the subtle end-ligation defects. Plasmid-based DNA repair

substrates are not able to access these beneficial chromatin effects and thus more sensitive to mild cNHEJ defects. Consistent with this hypothesis, Xlf-deficiency substantially reduced plasmids based V(D)J recombination[30,81,82], while largely dispensable for chromosomal V(D)J recombination[83–85]. Similarly, loss of DNA-PKcs or the T2609 cluster phosphorylation of DNA-PKcs have a significant impact on the end-ligation of plasmid substrates[65,70,86], but not chromosomal substrates[87,88] or *in vivo*[51,77,78,89]. Strikingly, in both Xlf-deficient or DNA-PKcs-deficient cells, loss of ATM kinase activity or chromatin bounded ATM substrates – H2AX, MDC1, and 53BP1 abolish the residual end-ligation on chromosomal susbstrate[85,87,88,90–94], supporting a critical role of chromatin and DNA damage response in promoting end-ligation. ATM kinase inhibition or deletion also abolishes SJ formation in murine cells carrying DNA-PKcs with alanine substitutions at the T2609A cluster[80], but not those with alanine substitutions at the S2056 cluster[77]. Furthermore, RAG has extensive C-terminal chromatin interaction domains that are required for chromatin V(D)J recombination, but not for episomal assay[26,95–99], suggesting RAG might also facilitate end-ligation through chromatin interaction. In addition to the availability of chromatin bounded DDR response and RAG interaction, the cell cycle could be another factor. While most episomal assays were performed in cycling cells when RAG and other nuclease were ectopically expressed throughout the cell cycle, chromosomal V(D)J recombination and V(D)J recombination *in vivo* occurs only in G1 arrested cells due to the transcription and post-translation regulation of RAG[24,100]. One notably difference is at the hairpin-opening. While Artemis is strictly required for the hairpin-opening during V(D)J recombination, several other endonucleases including MRE11 in complex with CtIP can open hairpin, including RAG generated hairpin in S or G2 phase of the cell cycle[18,101,102]. S and G2 phase cells also express other DNA repair factors involved in end-resection, and HR that are not available in G1 cells, plus the availability of sister chromatin nearby could introduce additional complexity and possibilities for DNA repair.

Second, IR sensitivity vs defects in V(D)J recombination. From a DNA repair point of view, RAG cleavage generates clean DNA ends that might be less vulnerable to end-ligation defects than complex DNA lesions including oxidized bases generated during IR[89,103–106]. Consistent with this hypothesis, Artemis is not required for SJ formation during chromosomal V(D)J recombination, but clear responsible for ~10% of complex ends generated by IR[33,53,107]. RAG also holds the SEs after cleavage and has extensive C-terminal chromatin interaction domains uniquely important for chromatin V(D)J recombination[22,23,26,108–112], which can also facilitate end-ligation. Moreover, IR sensitivity in proliferating cells reflects a complex response to DNA damage beyond simple DNA repair. For example, loss of TP53 or its regulators and targets (*e.g.*, ATM, ARF, CHK1, MDM2, P21, PUMA, and others) can all affect IR sensitivity in a cell-type and context-specific manner without directly affect DNA repair. This is particularly true for human cancer cell lines, which often carry complex genetic alterations. In this context, DNA-PKcs has been implicated in numbers of functions outside cNHEJ, including telomere biology[113,114], Golgi stability[115], DNA damage checkpoints[116], and others[117]. In certain human cells including HCT116 and 293T cells, loss of DNA-PKcs also leads to adaptive loss of ATM protein expression, which further modulates IR sensitivity and DNA repair[70,86]. Notably, ATM activity, measured by the phosphorylation of KAP1, seems intact if not

hyperactive in DNA-PKcs null, DNA-PKcs-T2609A, or *DNA-PKcs*$^{KD/KD}$ murine cells[80,118] (Crowe and Zha unpublished observations). Biochemical analyses have shown that DNA-PKcs can phosphorylate ATM kinase to inhibit ATM kinase activity[118]. A uniformed hypothesis regarding how DNA-PKcs protein or activity loss might lead to an adaptive decrease of ATM protein levels remain pending, would hold the key to understand the cross-talk between ATM and DNA-PKcs. Moreover, we and others recently showed that mouse models with an alanine substitution at the T2609 cluster succumb to bone marrow failure[79] with macrocytic anemia associated with ribosome biogenesis defects[78]. These and other cNHEJ independent functions of DNA-PKcs might be particularly important in understanding complex responses to radiation or other environmental insults beyond simple DSB repair.

Third, the role of DNA-PKcs in human vs in mice. It is noted early on, the abundancy of KU and DNA-PKcs, but not other cNHEJ factors, is nearly 50-fold higher in human cells than in mouse cells[77], suggests that KU and DNA-PK might have cNHEJ-independent functions in human cells. The high concentration of KU and DNA-PKcs could potentiate relative low-affinity interaction with structured RNA, including the telomerase RNA template, which might contribute to the prominent telomere abnormalities[119,120] in human cells lost KU or DNA-PKcs. Accordingly, while DNA-PKcs or KU null mice are viable, human cells, even cancer cells, cannot tolerate the complete and persistent loss of KU[120] or DNA-PKcs without adaptive changes of other genes, including reducing ATM protein levels[70,121]. Several human patients derived cell lines contain spontaneous mutations of DNA-PKcs are viable, but whether the residual DNA-PKcs protein or activity or adaptive changes are essential for their long-term culture is yet to be determined[54,56,122].

Finally, the difference between the kinase-dead and the phosphorylation site mutant proteins is not unique to DNA-PKcs. The expression of kinase-dead ATM also leads to different biological consequences than the alanine substitutions of its proposed auto-phosphorylation sites[123–127]. These genetic models demonstrate a structural role of DNA-PKcs that is modulated by its kinase activity, resulting in different phenotypes for loss of DNA-PKcs, expression of kinase-dead, and expression of an alanine substitution at the T2609 and/or S2056 clusters. The recent cryogenic electron microscopy structure of the DNA-bound and DNA-free forms of the DNA-PK holoenzyme uncovered an extended interface between KU and DNA-PK[128–131]. Several questions are pending, including the contribution of the proposed phosphorylation sites to the allosteric activation of DNA-PKcs, the exact mechanism by which DNA binding triggers KU-dependent activation of DNA-PKcs, the exact roles of the KU70 SAP domain and the KU80 C-terminal domain in cNHEJ and V(D)J recombination, and the interaction surface and the regulation between DNA-PKcs and Artemis. Collectively, this information would help us understand the dynamic movements that lead to DNA-PK activation and how DNA-PKcs caps the DNA ends and activates Artemis endonuclease.

2.3. The new cNHEJ factors, and their roles in cNHEJ and V(D)J recombination

In the past few years, several new cNHEJ factors have been described - PAXX and MRI - based on their interaction with KU and their essential role in V(D)J recombination in mice

or cells lacking XLF, a non-essential cNHEJ factor that is associated with mild lymphocyte development defects in mouse models[41,81,84]. PAXX binds to the core region of the KU heterodimer[132,133]. *Paxx*$^{-/-}$ mice develop normally with normal levels of mature lymphocytes, but Xlf/Paxx double deficient cells accumulate both CEs and SEs, suggesting a critical role of Paxx in end-ligation that is masked by Xlf[134–136]. Several models have been proposed to explain the role of XLF in cNHEJ, including the formation of XLF-XRCC4 filament that brings the two DNA ends together[5,137] and facilitating Lig4 re-adenylation[138]. We envision that the presence of XLF increases ligation frequency, thus indirectly compensates for the compromised cNHEJ in Paxx-deficient cells. Notably, Paxx shares some structural features with XLF[132,133]. Similarly, Mri also binds to KU and is essential for chromosomal V(D)J recombination in *Xlf*$^{-/-}$ cells[139]. While Mri promotes cNHEJ and V(D)J recombination in G1 arrested B cells, it is also thought to suppress cNHEJ mediated telomere fusion in S or G2 phase cells[140]. Exactly how MRI shifts between these two seemingly opposing functions remains unknown. In addition to KU, MRI also binds to ATM and the MRE11-RAD50-NBS1 (MRN) complex. MRI might mediate different functions by associating with different molecular complexes[139]. On this note, whether cell cycle or cyclin-associated kinases might play a role in regulating MRI association and function also needs to be tested. Notably, unlike LIG4, XRCC4, XLF, DNA-PKcs, and ARTEMIS, the mutations of which have been linked to human primary immunodeficiency[20,30,33,50,54,56,141], PAXX, and MRI be not mutated in immunodeficient patients and their connection to human immunodeficiency remain unknown.

2.4. The role of the DNA damage response in V(D)J recombination

DSBs generated during V(D)J recombination activate the DDR. Briefly, the MRN complex recruits and activates ATM kinase, which phosphorylates many substrates, including the histones H2AX flanking the DNA, break at S139 to form γH2AX[142]. MDC1 directly binds to γH2AX[143–145] and recruits the E3 ubiquitin ligases RNF8 and RNF168, which further modify H2A and eventually leads to the recruitment of 53BP1[7]. ATM and the downstream factors H2AX and 53BP1 are not required for lymphocyte development, but in mouse models, their absence causes a mild reduction in surface TCRβ/CD3ε levels in CD4+CD8+ double-positive T cells[146–148] and the accumulation of unrepaired RAG-dependent DSBs in ~4% of Atm-deficient B cells[149]. The need for sequential V(D)J recombination at the TCRα/δ[150] likely contributes to its hypersensitivity to DNA repair defects (Table 1). Correspondingly, loss of ATM, H2AX, or 53BP1 does cause major development blockade associated with V(D)J recombination in the majority of the Ig and TCR loci(Fig. 1 and 2C). Using an inversional chromosomal V(D)J recombination substrate, Sleckman and colleagues showed that MRN and ATM prevent hybrid joins – the aberrant ligation of a CE with a SE during inversional V(D)J recombination[151,152] - providing a mechanism for the mild lymphocyte development defects associated with ATM-deficiency[149](Fig. 2C). Interestingly, ATM and ATM kinase activities are required for SJ formation in DNA-PKcs null cells[87,88], supporting overlapping roles between ATM and DNA-PKcs kinase activity. Moreover, in cells and mice lacking XLF, a non-essential cNHEJ factor, ATM kinase activity, and its substrates H2AX and 53BP1 are required for SJ formation and end-ligation[85,91,92] also, providing genetic evidence for an important role of the DDR in V(D)J recombination. In this context, whether other DDR factors are also required for V(D)J recombination in the

sensitized XLF-deficient background and whether DDR factors have an additional role in V(D)J recombination beyond ligation (such as end-protection) remain to be tested. How the chromatin-based DDR network interacts with the epigenetic environment at the TCR and Ig loci required for V(D)J recombination remains unclear. It is possible that the epigenetically active TCR or Ig loci are poised to enhance repair and prevent mis-repair through higher order topologically associated domains (TADs)[153] to promote effective V(D)J recombination.

3. DNA repair during Immunoglobulin Class Switch Recombination

Ig CSR generates antibodies with different isotypes and thus different effector functions. B cell-specific activation-induced cytidine deaminase (AID) initiates CSR by converting cytosine to uracil in the transcribed S regions preceding each set of constant region coding exons[154]. These mismatches are processed by both base excision repair and mismatch repair machinery and are eventually converted to DSBs in proliferating B cells. In the second phase, DSBs in the upstream IgM switch region (Sμ) are joined with DSBs in the downstream switch region (*e.g.*, Sγ1 for IgG1) to complete IgH isotype switching (Fig. 1) and generate antibodies with different isotypes. The DSB repair that completes CSR is mediated by the cNHEJ and the Alt-EJ pathway with the help of ATM kinase and its chromatin substrates H2AX, 53BP1, etc.

3.1. The role of cNHEJ in CSR

To study the role of cNHEJ in CSR, B cells can be generated in cNHEJ-deficient backgrounds via the introduction of germ-line knock-in alleles harboring productive V(D)J rearrangements at both the IgH[155] and Igκ[156] loci. Unexpectedly, Ku70-, Xrcc4- or Lig4-deficient mature B cells generated using this approach[157–159] and germline Xlf-deficient B cells[82,84] all have significant residual CSR (25–50% of the WT levels) that is mediated by an Alt-EJ pathway biased towards MH (Table 1). Expression of kinase-dead DNA-PKcs causes severe CSR defects, phenocopies Xrcc4- or Lig4-deficiency[106] (Table 1). In contrast, *DNA-PKcs* or *Artemis* null B cells have at most moderate defects in CSR efficiency that is detectable only by sensitive IgH FISH analysis[160,161], consistent with the limited impacts of DNA-PKcs or Artemis deletion in end-ligation (Table 1). The recently developed high throughput genome-wide translocation sequencing (HTGTS)[162] can isolate thousands of CSR junctions. CSR junctions recovered from $Xrcc4^{-/-}$ and $DNA\text{-}PKcs^{KD/KD}$ B cells are enriched for MHs, with nearly 50% of junctions containing 2–3 nt MHs, in contrast to 20–25% in WT cells[106]. Perhaps most surprising is that the CSR junctions from $DNA\text{-}PKcs^{-/-}$ B cells with nearly 90% of WT levels CSR are also equally enriched for MHs[106], suggesting MHs facilitate CSR. But whether this MH-mediated CSR in $DNA\text{-}PKcs^{-/-}$ B cells depends on Alt-EJ or LIG4-mediated cNHEJ remain to be determined. Two features of CSR might contribute to the robust residual CSR in cNHEJ deficient cells that prefer the MH-mediated end-joining – the highly repetitive and GC rich switch region that is ideal for MH-mediated repair[163] (Fig. 3A) and the S/G2 phase of the cell cycle that allows resection and promotes end-annealing. The loss of the newly discovered cNHEJ factor PAXX does not affect CSR efficiency[136] and whether PAXX or even Artemis affects CSR junctions remains to be determined.

3.2. The role of Alt-EJ in Class switch recombination

The robustness of Alt-EJ mediated CSR provides a unique tool to study the Alt-EJ pathway. In contrast to cNHEJ that ligates both strands of the DNA double helix simultaneously, the Alt-EJ pathway uses MH-mediated annealing to convert one DSB into two single-strand nicks (Fig. 3B). Mammalian cells have three DNA ligases, LIG1, LIG3, and LIG4, which repair the phosphodiester backbone in each DNA strand in an adenylation-dependent manner. Each strand ligation requires re-adenylation of the ligases[138]. LIG4 can potentially form higher-order oligomers through its interaction with the XRCC4-XLF filament[137,164], which might explain the unique ability for LIG4 to mediate double-strand break repair. The conversion of a DSB to two single-strand nicks bypasses the requirement for LIG4 and allows the single-strand DNA ligases - LIG3 in complex with XRCC1 or LIG1 – to complete the Alt-EJ mediated CSR[165]. End-resection, which is required for MH-mediated repair to expose flanking MHs, is also evident in *Xrcc4*$^{-/-}$, *DNA-PKcs*$^{-/-}$ and *DNA-PKcs*$^{KD/KD}$ B cells[106]. However, it remains unknown whether end-resection is required for Alt-EJ mediated CSR in the highly repetitive switch region. Nevertheless, the similar CSR efficiency and the MH and end-resection patterns in *Xrcc4*$^{-/-}$ and *DNA-PKcs*$^{KD/KD}$ cells suggest that the presence of kinase-dead DNA-PKcs does not block the Alt-EJ pathway[106]. Moreover, the deletion of *Ku70* in *Lig4*$^{-/-}$ B cells reduces the bias toward MHs[166,167], suggesting there might be more than one Alt-EJ pathway – the one requires long MH and the one that does not. Also, pol θ expression increases in activating B cells[168] and might contributes to Alt-EJ through templated nucleotide insertions. Although *Polq*$^{-/-}$ B cells have normal CSR efficiency[169], the percentage of recovered junctions with insertions is significantly lowered[170]. Interestingly, *Polq*$^{-/-}$ B cells also show increased IgH-Myc chromosomal translocations[170], suggesting a role of pol θ in suppressing MH mediated translocations. The genetic requirement for Alt-EJ mediated CSR is still being investigated. Loss of Alt-EJ factors, including XRCC1, an obligatory partner of Lig3 in the nucleus[171], PARP1, or POLQ, all did not compromise CSR efficiency alone[165,169,172,173]. Loss of DNA-PKcs[103–105,161,174] or DNA-PKcs phosphorylation at the T2609 cluster[89] has a mild effect on IgG1 CSR efficiency *in vitro*. But the CSR junctions recovered from the *DNA-PKcs*$^{-/-}$ or *DNA-PKcs*$^{5A/5A}$ mice are highly enriched for MH[89,106]. The results suggest that MH-mediated end-joining could be quite robust during CSR. Given the much more severe CSR defects in *Lig4*$^{-/-}$ B cells, the end-ligation that generates these small MH are likely including contributions from both cNHEJ pathway and the Alt-EJ pathway. The exact contribution of Alt-EJ to physiological CSR in cNHEJ-proficient cells also remains to be determined.

3.3. The role of the DNA damage response in CSR.

In contrast to V(D)J recombination that is only mildly affected by DDR-deficiency or in sensitized backgrounds, CSR highly depends on the DDR. CSR is significantly (>50%) impaired in cells deficient in ATM[175–177] or its downstream targets H2AX[178,179], MDC1[143,180], RNF8, RNF168, or 53BP1[75], or even PTIP[181,182], REV7(gene name MD2L2)[183,184], SHIELDIN complex[183,185,186], or RIF1[187,188]. which prevents end-resection downstream of 53BP1(Fig. 3C). ATM kinase inhibitors or expression of kinase-dead ATM have similar impacts on CSR as Atm loss[123], suggesting that ATM contributes to CSR mainly through its kinase activity. Notably, although 53BP1 has a similar, if not a

weaker, role in general DSB repair and irradiation resistance than ATM or H2AX, loss of 53BP1 leads to a 95% reduction of CSR that is much more prominent than all other repair factors[179,180,189]. It has been proposed that overreaction in the 53BP1-deficient cells exaggerates the CSR defects[189]. Moreover, 53BP1 is also thought to have a role in CSR upstream of DSB repair that is mediated by its downstream effector protein PTIP, which regulates the histone acetylation complex MLL2/3[181,182]. More recently, it has also been proposed that 53BP1 ensures that the orientation of CSR junctions to favor productive CSR beyond end-ligation[181]. Recent HTGTS sequence analyses of CSR junctions recovered from ATM-deficient or H2AX-deficient cells also show increased MHs[190], suggesting the repetitive sequence in the switch region might poise CSR for MH-mediated joining independent of specific repair pathways. In the future, systematic analyses of CSR junctions with HTGTS might uncover additional regulators of Alt-EJ at the chromosomal levels.

4. Closing remarks

Efficient DNA repair during V(D)J recombination and CSR is not only important for lymphocyte development but also plays a critical role in suppressing oncogenic translocation. Indeed, the majority of recurrent chromosomal translocations in human lymphoid malignancies involve the Ig or TCR loci and arise from aberrant DSB repairs during V(D)J recombination or CSR[191]. While defects in the cNHEJ pathway often cause severe combined immunodeficiency (SCID)[192], defects in the DDR, especially in ATM kinase which also has a checkpoint function, often lead to early-onset lymphoma and leukemia, providing a model to study the mechanism of chromosomal translocations[147,193,194].

In summary, V(D)J recombination highly depends on core cNHEJ factors, while the DDR has more prominent roles in CSR. Two factors might contribute to this difference; the postcleavage complex formed by RAG and the strict G1 phase repair during V(D)J recombination. Moreover, the repetitive switch region sequences and the proliferative nature of B cells undergoing CSR render Alt-EJ very robust during CSR and provide unique opportunities to study the genetic requirement for Alt-EJ at the physiological level. On the other hand, the hairpin structure and exclusive G1 phase repair of V(D)J recombination provide an opportunity to study end-processing and the regulation of DNA-PKcs during end-joining. With the development of high-throughput sequencing methods and chromosomal V(D)J recombination reporters, significant strides have been made in understanding the regulation and function of cNHEJ and the DDR and their interaction *in vivo.* With the development of CRISPR genetic screens, we expect that additional DDR factors can be identified based on their role in V(D)J recombination and CSR, and further expand our knowledge of DNA double-strand break repair networks.

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Abbreviations

Alt-EJ	Alternative end-joining
CE	coding end
CJ	coding join
cNHEJ	classical non-homologous end-joining
CSR	Class Switch Recombination
DDR	DNA damage response
DSB	DNA double-strand break
HR	homologous recombination
HTGTS	High throughput genome-wide translocation sequencing
IgH	Immunoglobulin Heavy Chain
MH	microhomology
MMEJ	microhomology-mediated end-joining
RSS	recombination signal sequences
SE	signal end
SJ	signal join

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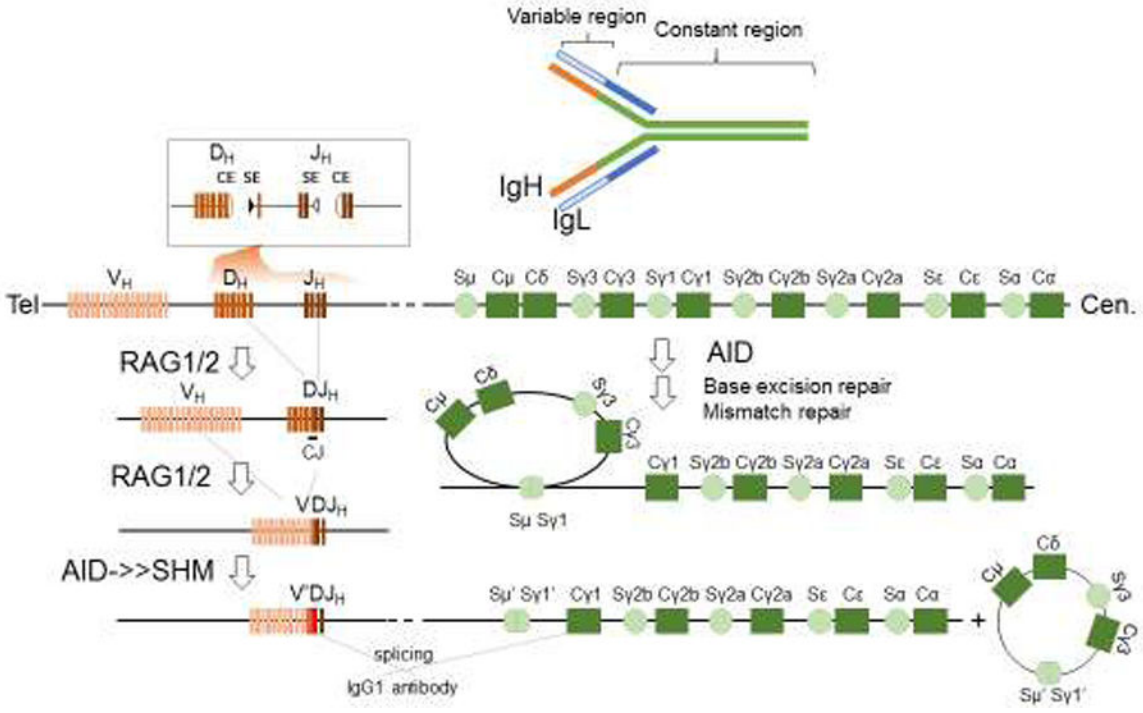

Figure 1. The overview of V(D)J recombination and class switch recombination

The Immunoglobulin (Ig) gene product, an antibody, is shown at the top. An antibody is formed by a pair of Ig Heavy chain gene products (IgH, in orange and green) and a pair of Ig light chain (IgL, in white and blue). The V(D)J recombination (on the lower left) assembles the variable gene exon that encodes the antigen-specific portion of the antibody (orange) from individual V, D or J gene segments in a two-step process, D to J first, then V to DJ. The reaction is initiated by RAG endonucleases, which introduces DNA double-strand breaks. An insert above shows the pair of RAG cleavage products at Dh and Jh with the hairpin coding ends and blunt signal ends adjacent to RSSs (triangles). Upon ligation by the cNHEJ pathway, the intermediate sequence is removed and the participating V, D, and J segments are fused to form the variable region exon that is spliced with downstream constant region exons (on the right) to form the IgH. While we only depict the IgH here, V(D)J recombination occurs in all 3 Ig (IgH, Igλ, and Igκ) and 4 T cell receptor (TCRσ/δ, TCRγ, TCRβ) gene loci. In naïve B cells, the IgH undergoes two additional modifications initiated by activating induced deaminase (AID). Somatic hypermutation (SHM) introduces point mutations in the variable region exon without creating DNA double-strand breaks. Class switch recombination (CSR) occurs in the constant region (on the right) and joins DNA double-strand breaks from two different switch (S) regions and effectively replaces the initially expressed IgM constant region (Cμ) with a downstream constant region, such as the Cγ1 for IgG1 depicted in the illustration. The constant region exons dictate the effector function of the antibody. CSR only occurs in the IgH locus of B cells, not in IgL or TCR loci. In contrast to RAG, which directly introduces DNA breaks, AID introduces U:G mismatches, which are processed by the base excision repair and mismatch repair pathways to generate mutations for somatic hypermutation (SHM) and DNA double-strand breaks for CSR.

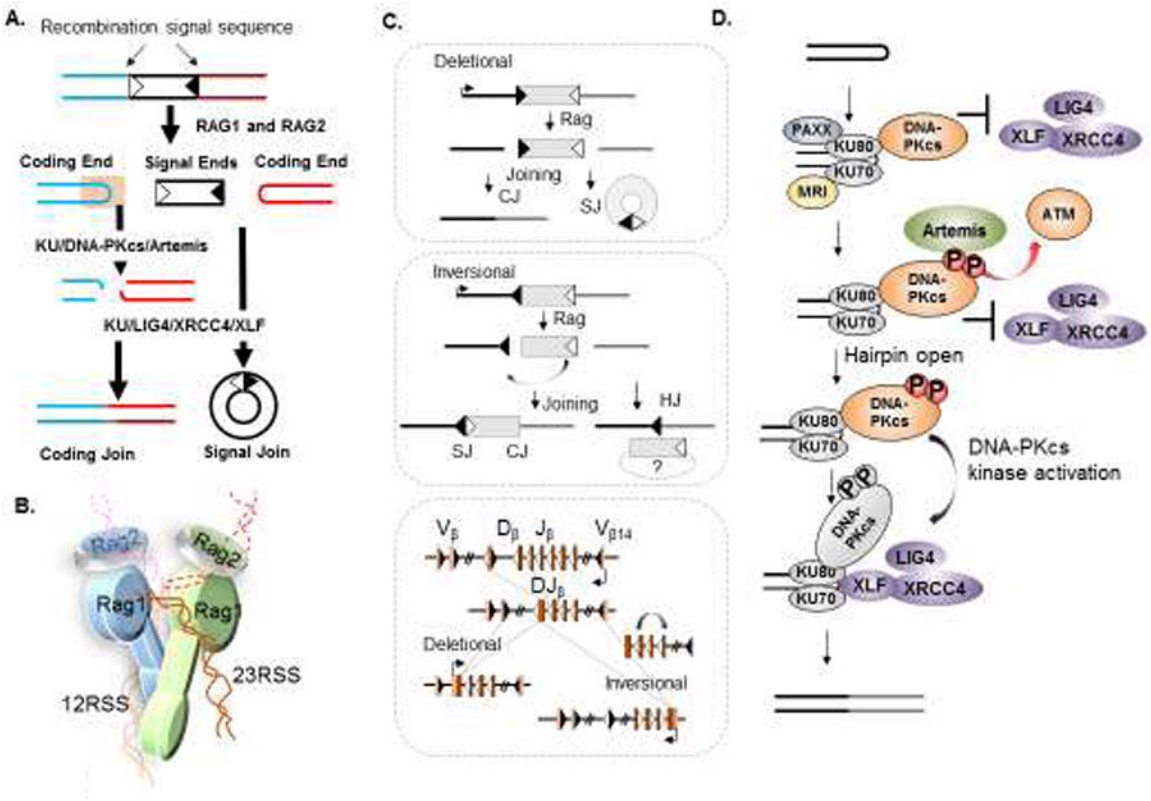

Figure 2. The features of V(D)J recombination and the cNHEJ pathway

(A) RAG1/2 endonuclease cleavage occurs after synapsing two compatible RSSs (with different spacer length, 12 and 23bp, illustrated as white or black triangles) together and generates a pair of 5' phosphorylated blunt signal ends (SE) and a pair of covalently sealed hairpinned coding ends (CE) (red and blue hairpins). The two SEs can be directly joined by KU together with the LIG4-XRCC4-XLF complex to form a signal join (SJ). The two CEs have to be opened by KU-DNA-PKcs and Artemis endonuclease before they can be ligated to form a coding join (CJ). (B) A cartoon of the RAG post-cleavage complex shows two RAG1 and two RAG2 proteins synapsing two compatible RSSs (with 12 bp, 12RSS or 23 bp, 23RSS spacers) together and also holding the resulting SEs (solid lines, open) and CEs (dash lines, hairpinned) in close proximity to facilitate ligation[22,108,195]. The precise position of the CEs is yet to be firmly determined. (C) V(D)J recombination can occur in a deletional (upper) or inversional (middle) configuration depending on the relative orientation of the participating RSSs (open or filled triangles). The vast majority of V(D)J recombination occurs in the deletional configuration (*e.g.*, IgH in Figure 1) with the two RSSs facing each other, and the recombination removing the SJ-containing internal sequence as an excised circular DNA (top). In rare cases, such as the TCRβ locus diagrammed at the bottom, the V(D)J recombination occurs between a pair of RSSs in the same orientation, and, as such, the recombination leads to the inversion of the internal sequence (grey box) to form an SJ on the chromosome in addition to the CJ (bottom left of the middlebox). In the case of ATM deficiency[151], the internal sequence is lost in ~50% of the cases, and, as such, the CE and SE on the genome join together to form a hybrid join (HJ) accompanied by the deletion of the internal sequence, indicated on the bottom right of

the middlebox. A diagram of the murine TCRβ locus, including the Vβs with deletional rearrangements and the Vβ14 that undergoes inversional V(D)J recombination,[12] is shown at the bottom. (D) A cartoon illustration of non-homologous end-joining at a CE. The KU heterodimer binds to the hairpin sealed CEs, and then recruits DNA-PKcs, which, upon phosphorylation by itself or by ATM kinase, serves as a platform to recruit and activate Artemis endonuclease. In the absence of kinase activation, DNA-PKcs at the ends precludes the DNA ligation complex from accessing the ends as well. The successful activation of DNA-PKcs triggers a conformational change that then allows end ligation by the LIG4-XRCC4-XLF complex.

A.

Chr12	Murine Sμ Sequence segment	GC%
113,424,221	CCCAGCTCAACTCAAAACATCCCGGCTCAC	57%
113,424,251	CCCAGTTCACCTCAGCTCAGCTCACTCCAGCTCA	59%
113,424,285	CCCAGCCCAGTTCACCCTAGCTCAGCTCAC	63%
113,424,315	CCCAGCTCAGCTCAGCTCAC	65%
113,424,335	CCCAGCTCAGCTCAGCTCAC	65%
113,424,355	CCCAGCTCAGCTCA	64%

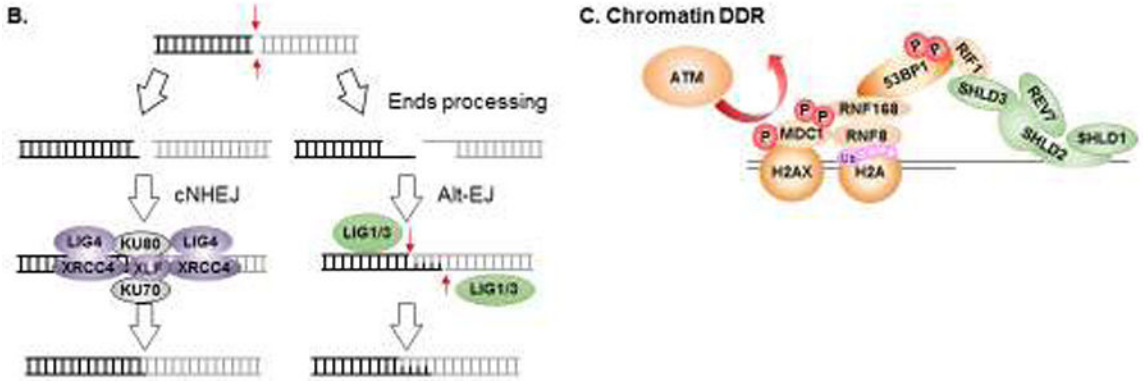

Figure 3. The features of class switch recombination and the DNA damage response

(A) The switch region sequence is highly repetitive and GC rich. A collection of representative murine Sμ regions, (chr12 113424221–113424368, mm10) is shown. The genomic coordinates of the first bp of each sequence segment are listed in the first column and the GC% within the segment is noted in the last column. The overall GC% is 61%. (B) A cartoon of cNHEJ vs Alt-EJ during CSR. The cNHEJ mediated by KU heterodimers and the LIG4-XRCC4-XLF ligation complex directly ligate both strands of a double-strand break. Alternatively, annealing between complementary single-strand overhangs converts one double-strand break into two single-strand nicks, which can then be ligated by LIG3 or LIG1 through Alt-EJ. (C) A simplified cartoon illustrating the main DDR players that have been implicated in CSR. Upon DNA damage, ATM kinase phosphorylates histone H2AX to form γH2AX, which recruits MDC1. MDC1 subsequently recruits E3 ubiquitin ligases RNF8 and RNF168 to modify adjacent H2A. Histone Ub modifications, together with H4K20me2, recruit 53BP1 and leads to hyperphosphorylation of 53BP1. This phosphorylated 53BP1 recruits RIF1 and the SHIELDIN (SHLD1–3 and REV7) complex. Among the SHIELDIN complex, SHLD2 serves as a scaffold. The N-terminus of SHLD2 binds to SHLD3 and REV7, while the C-terminus of SHLD2 interacts with SHLD1 and single-strand DNA. These factors work together to prevent excessive end-resection and promote productive CSR.

Table 1

Summary of V(D)J recombination and CSR phenotypes associated with major cNHEJ and DDR factors.

	Gene	Type of Mutation	IR sensitivity	Mice	V(D)J recombination				Class switch recombination			Ref.
					Chromosomal		Episomal/Plasmid		IgG1 % of Ctrl	Resection	MH at junctions	
					SJ (blunt)	CJ (hairpin)	SJ (blunt)	CJ (hairpin)				
non-homologous end-joining	KU70	Null	++++	sick	↓↓↓	↓↓↓	↓↓↓	↓↓↓	~30%	N.D.	↑	45,167,196–198
	KU80/86	Null	++++	sick	↓↓↓	↓↓↓	↓↓↓	↓↓↓	~30%	N.D.	↑	43,44,135,167,199,200
	XRCC4	Null	++++	lethal	↓↓↓	↓↓↓	↓↓↓	↓↓↓	~30%	↑↑	↑↑	157,201,202
	LIG4	Null	++++	lethal	↓↓↓	↓↓↓	↓↓↓	↓↓↓	~30%	↑↑	↑↑	46,159,166,203–206
	XLF	Null	+++	live	–	–	↓↓	↓↓	~50%	N.D.	↑↑	81,83,84,207
	DNA-PKcs	Null	+++	live	_[1]	↓↓↓	↓	↓↓↓	90%	↑↑	↑↑	51,52,103–105,208
	ARTEMIS	Null	++	live	–	↓↓↓	–	↓↓↓	>90%	N.D.	↑↑	53,90,209–213
	DNA-PKcs	KD	++++	lethal	↓↓↓	↓↓↓	↓↓	↓↓↓	30%	↑↑	↑↑	55,106
	DNA-PKcs	T2609A	++	sick	–	–	↓	–	>90%	↑	↑↑	70,72,73,76,78–80,89,214
	DNA-PKcs	S2056A	+	live	–	–	↓	↓	100%	–	–	76,77,214
	PAXX	Null	+	live	–	–	–	–	100%	–	–	132–136,215–217
	MRI/CYREN	Null	+	live	–	–	N.D.	N.D.	~90%	N.D.	N.D.	139,140,218
DNA damage response	ATM	Null	++++	live	–	↓[2]	–	–	50%	↑	↑↑	85,123,148,190,219–224
	ATM	KD	++++	lethal	–	↓[2]	N.D.	N.D.	50%	N.D.	N.D.	123,151,152
	ATM	S1987A	_[4]	live	–	–	N.D.	N.D.	100%	N.D.	N.D.	125,126
	H2AX	Null	+++	live	–	–	N.D.	N.D.	~30%	N.D.	↑↑	178,179,190,225–228
	MDC1	Null	+++	live	–	–	N.D.	N.D.	~50%	N.D.	N.D.	94,143,145,229
	53BP1	Null	++	live	–	–	N.D.	N.D.	5%	↑↑	↑↑	146,188–190,230,231
	RIF1	Null	+++	lethal	–	–	N.D.	N.D.	10%	↑↑	↑↑.	187,188,190,232,233
	REV7	Null	+++	sick	–	–	N.D.	N.D.	10%	↑↑	N.D.	183,234–236
	SHLD1/2/3	Null	++	N.D.	–	–	N.D.	N.D.	10%	↑↑	N.D.	183,185,186
	PTIP	Null	++	lethal	N.D.	N.D.	N.D.	N.D.	~50%	N.D.	N.D.	181,237–241

	Gene	Type of Mutation	IR sensitivity	Mice	V(D)J recombination				Class switch recombination			Ref.
					Chromosomal		Episomal/Plasmid		IgG1 % of Ctrl	Resection	MH at junctions	
					SJ (blunt)	CJ (hairpin)	SJ (blunt)	CJ (hairpin)				
Combinations	XLF&ATM	Null	++++	live	↓↓↓	↓↓↓	↓↓	↓↓	30%	N.D.	N.D.	85
	XLF&H2AX	Null	++++	lethal	↓↓↓	↓↓↓	N.D.	N.D.	N.D.	N.D.	N.D.	85
	XLF&MDC1	Null	++++	lethal	↓↓	↓↓	N.D.	N.D.	N.D.	N.D.	N.D.	94
	XLF&53BP1	Null	++++	live	↓↓↓	↓↓↓	N.D.	N.D.	N.D.	N.D.	N.D.	91,92
	XLF&ART	Null	++++	live	−	↓↓↓	N.D.	↓↓↓	N.D.	N.D.	N.D.	90
	XLF&DNA-PKcs	Null	++++	sick	↓↓	↓↓↓	N.D.	↓↓↓	~50%	N.D.	N.D.	90
	XLF&PAXX	Null	++++	lethal	↓↓↓	↓↓↓	N.D.	N.D.	N.D.	N.D.	N.D.	134–136,242,243
	XLF&MRI	Null	++++	lethal	↓↓↓	↓↓↓	N.D.	N.D.	N.D.	N.D.	N.D.	139
	DNA-PKcs&ATM	Null	++++	lethal	↓↓↓	↓↓↓	↓↓	↓↓↓	~20%	N.D.	N.D.[3]	70,87,88,93,244,245
	DNA-PKcs&ATM	T2609A/ Null	N.D.	N.D.	↓↓↓	↓↓↓	↓	↓	N.D.	N.D.	N.D.	65,76,80
	DNA-PKcs&ATM	S2056A/ Null	N.D.	Live*	−	−	↓	↓	50%	N.D.	N.D.	65,76,77

N.D., not determined; −, no change;

[1] reduced fidelity;

[2] Hybrid join formation;

[3] increased insertion;

[4] IR sensitive in human cells;

* unpublished data.