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Review

Review

Alternative end-joining in BCR gene rearrangements and translocations

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Abstract

Programmed DNA double-strand breaks (DSBs) occur during antigen receptor gene recombination, namely V(D)J recombination in developing B lymphocytes and class switch recombination (CSR) in mature B cells. Repair of these DSBs by classical end-joining (c-NHEJ) enables the generation of diverse BCR repertoires for efficient humoral immunity. Deletion of or mutation in c-NHEJ genes in mice and humans confer various degrees of primary immune deficiency and predisposition to lymphoid malignancies that often harbor oncogenic chromosomal translocations. In the absence of c-NHEJ, alternative end-joining (A-EJ) catalyzes robust CSR and to a much lesser extent, V(D)J recombination, but the mechanisms of A-EJ are only poorly defined. In this review, we introduce recent advances in the understanding of A-EJ in the context of V(D)J recombination and CSR with emphases on DSB end processing, DNA polymerases and ligases, and discuss the implications of A-EJ to lymphoid development and chromosomal translocations.

Key words classical nonhomologous end-joining, alternative end-joining, V(D)J recombination, chromosomal translocation, DSB end resection, endonuclease

Introduction

DNA double-strand breaks (DSBs) are the most lethal form of DNA damages that can occur to our genome, as a single unrepaired or misrepaired DSB can lead to the activation of cell cycle checkpoint arrest and cell death. DSBs can arise from constant assaults by environmental factors and cellular metabolites, and from programmed cellular processes during antigen receptor gene diversification in developing B and T lymphocytes in the context of V(D)J recombination and class switch recombination (CSR) [1]. The timely and proper repairs of these DSBs in developing B/T cells are absolutely essential for the generation of a diverse repertoire of antigen receptors, and prevention of lymphoid malignancies in the form of B and T cell leukemias and lymphomas. As such, much of our knowledge of general DSB repair mechanisms has come from studying the DSB processes in lymphocyte development.

Eukaryotic cells repair DSBs mainly by two major pathways: nonhomologous end-joining (NHEJ) and homologous recombination (HR). The repair by HR requires DNA templates homologous to sequences around the DSB ends. HR is initiated by the 5′-3′ nucleolytic degradation of both broken ends, a process termed DSB end resection, to expose single-stranded DNA (ssDNA) overhangs [2]. The ensuing strand invasion by base-paring between ssDNA and template DNA sequences leads to the formation of the three-stranded D-loop structure that migrates along and copies the template information. As a result, HR often leads to an error-free repair of DSBs using available sister chromatids in the S and G2 phase of the cell cycle [3]. On the contrary, DSB repair by NHEJ simply religates broken DNA ends with minimal sequence deletion or insertion, and thus is error-prone and can operate in all phases of the cell cycle [4] (Figure 1).

Recent years had witnessed the discovery of a different DSB end-joining pathway (or pathways) called alternative end-joining (A-EJ) in various model systems (Figure 1). A-EJ products usually exhibit increased deletion of sequences around the break sites and higher usage of short homology sequences termed microhomology (MH) in the junctions. This feature often leads to a spontaneous equation of A-EJ to microhomology-mediated end-joining (MMEJ). However, although A-EJ is biased toward heavier use of MH, MH is not an exclusive feature of A-EJ, as c-NHEJ proficient cells also utilize certain amounts of MH (*e.g.*, B cells undergoing class switching)

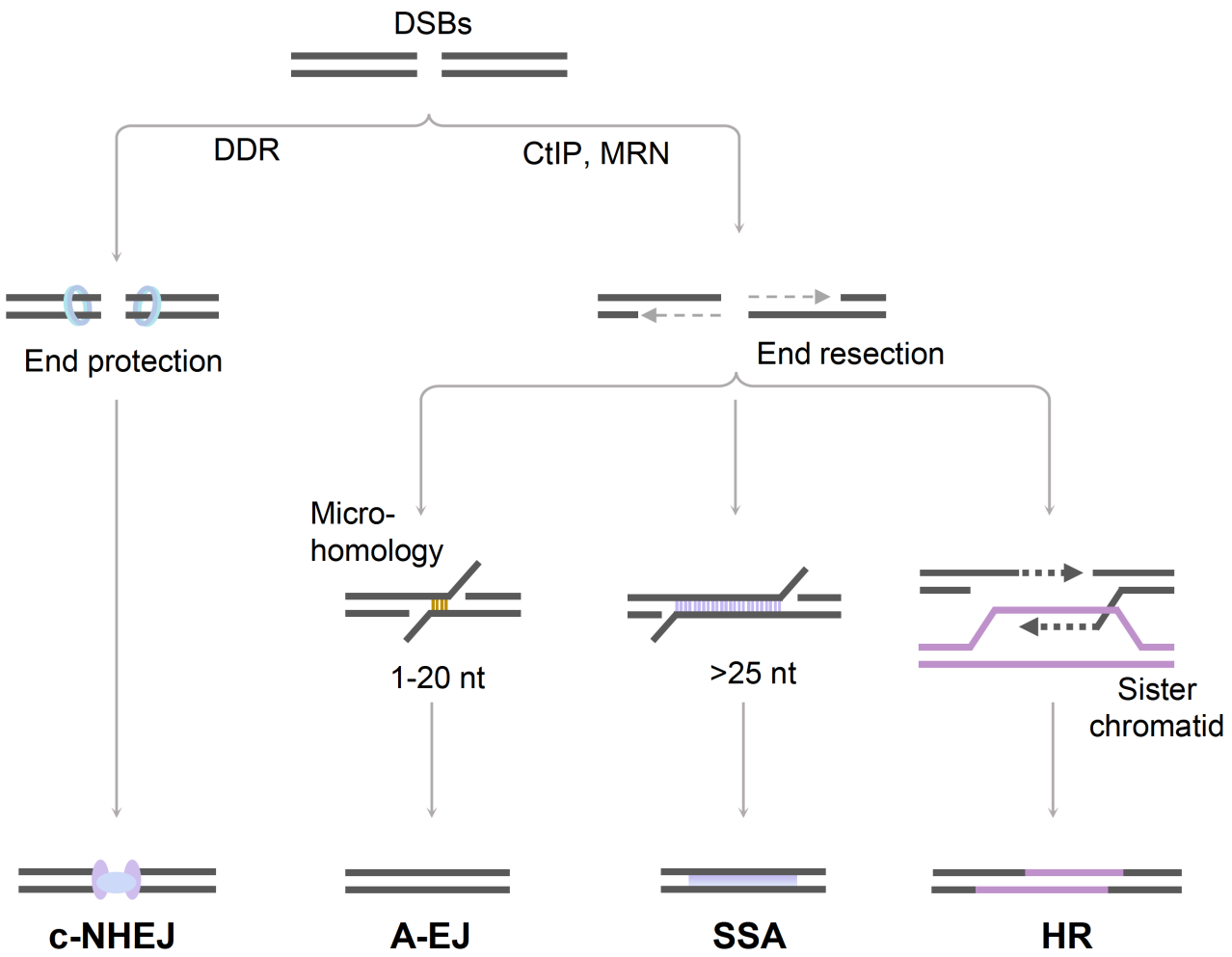

Figure 1. The DSB repair pathways in eukaryotic cells NHEJ re-ligates broken DNA ends with the help of DDR factors binding to and protecting ends from nucleolytic degradation. NHEJ operates in all phases of the cell cycle. On the contrary, HR is only active in S/G2 phase of the cell cycle due to requirement for DSB end resection-generated ssDNA for homology searching and invasion. SSA and A-EJ also require DSB end resection and homology sequence annealing. SSA and A-EJ mainly differ by homology length requirement and likely in essential components after annealing.

[5,6], and c-NHEJ-deficient B cells also contain direct end-joining. Thus, MMEJ only represents a portion of, but does not overlap entirely with A-EJ [7]. It is thus more appropriate to define A-EJ as end-joining events occurring independent of classical NHEJ factors. Recent studies revealed that A-EJ shares certain steps and features with HR [4,8], and several factors have been shown to be uniquely required for A-EJ but not c-NHEJ.

In this review, we introduce these recent insights into the major steps of A-EJ with a specific focus on lymphocyte development and oncogenic chromosomal translocations.

V(D)J Recombination and CSR during B Cell Development

B cell development is divided into two separate stages that take place in distant lymphoid organs. The early development in the bone marrow in the absence of foreign antigen encounter generates a diverse naïve B cell receptor (BCR) pool by the process of V(D)J recombination. BCR, the membrane-bound form of an antibody, comprises two identical heavy chains and light chains that are encoded by the immunoglobulin heavy chain gene (*IgH*) and the light chain genes (*IgL*), respectively. The 5′ portion of the mouse *IgH* locus is composed of variable (V), diversity (D) and joining (J) exons, and a successful V(D)J recombination brings together one of V, D and J exons each and recombines them in a cut-and-paste fashion to encode the variable region of the heavy chain of an antibody (Figure 2A). Similarly, V-J recombination on the *IgL* loci generates the light chain of an antibody [9]. V(D)J recombination is initiated by the RAG recombinase complex (Rag1 and Rag2) that generates site-specific DSBs at unique sequences adjacent to V, D and J exons termed recombination signal sequences (RSS). Recognition of RSS by Rag and accessory proteins facilitate the synapsis between remote V and D, J sequences for coordinated cleavage of DNA right at the border of exon and RSS [9]. Rag1 nicks the RSS and leaves a free 3′-OH that subsequently attacks the phosphodiester bond on the opposing strand to form a hairpin structure. Endonuclease Artemis is then activated through phosphorylation by the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) to cleave the hairpin structure, enabling non-templated nucleotide additions by terminal deoxynucleotidyl transferase (TdT) [9]. The modified ends are ligated by the DNA

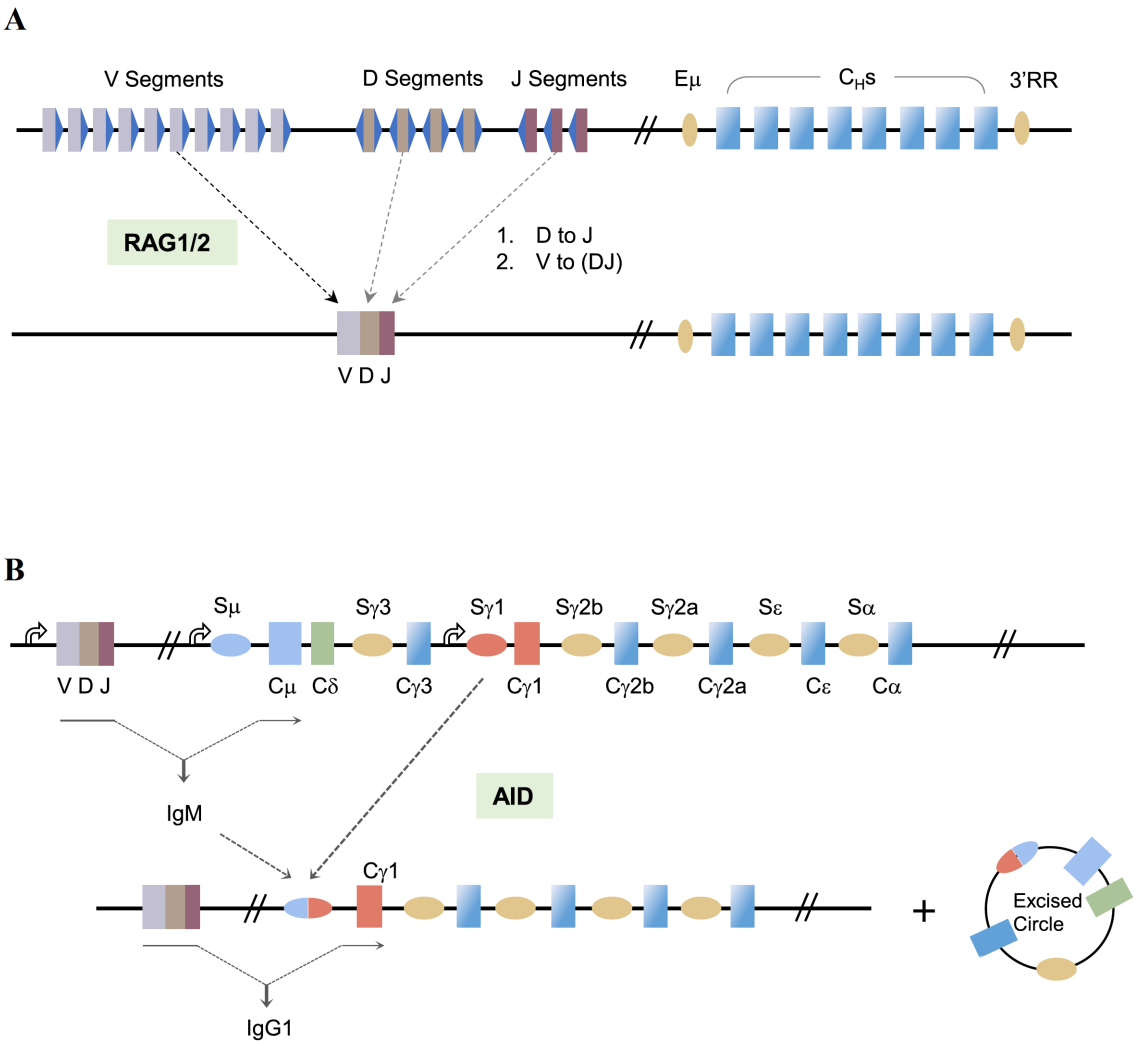

Figure 2. BCR gene rearrangements by V(D)J recombination and class switch recombination (A) Overview of V(D)J recombination. The mouse *IgH* locus contains multiple V, D, and J exons, and RSS is located adjacent to each coding segment. RAG1/2 initiates cleavage right at the RSS that is converted into DSBs on the coding end, and joining D to J followed by V- to DJ assembles a full exon for the variable region of an antibody. (B) Overview of mouse *IgH* class switch recombination. Stimulation of mature B cells by cytokines and ligands turns on AID expression and germline transcription of specific S regions, which facilitates targeting of AID to initiate S region DSBs. End-joining of the donor Sμ DSB and downstream S region DSB juxtaposes downstream constant exons to the assembled VDJ exon to express a different isotype of antibody without altering its specificity.

Lig4/XRCC4 complex with the help of Ku70/Ku80 complex [10] (see detail below).

Class switch recombination takes place when mature B cells are activated by foreign antigens in the germinal center or in vitro by mitogens and cytokines. CSR diversifies the effector function of an antibody through replacing the originally expressed IgM constant gene with a different one without altering its antigen binding specificity [11]. The 3′ side of mouse and human *IgH* loci contains several genes encoding constant regions for IgM, IgD, IgG, IgE and IgA isotypes. Except IgD each gene is independently transcribed from an (I) promoter that is followed by a long and repetitive intronic region called switch (S) region. When activated, B cells initiate the expression of activation-induced cytidine deaminase (AID) that recognizes the R(A/G)GY(C/T)W(A/T) motifs (most favorably, the AGCT motif) within specific S regions. Deamination of cytosine in these motifs by AID leads to its conversion to uracil and thus introduces a U-G mismatch. Subsequent actions by uracil DNA glycosylase (UNG) to remove the mismatched uracil, and by class II apurinic/apyrimidinic endonuclease (APE) to cleave the phosphodiester bond 5′- to the AP site generate a nick at the DNA backbone [11]. Because RGYW motifs are so dense in the switch IgM (Sμ) and downstream switch regions, the combined actions by AID/UNG/APE result in proximate DNA nicks that resemble double-strand breaks. Finally, the joining of Sμ and downstream S region DSBs by the classical NHEJ pathway in a deletion-preferred fashion [12] juxtaposes the downstream constant region with the assembled V(D)J region, leading to the expression of an antibody with altered effector functions (Figure 2B) [11,13]. AID-initiated S region DSBs also trigger activation of the master DNA damage response (DDR) kinase Ataxia telangiectasia-mutated (ATM), which phosphorylates a series of downstream targets (histone variant H2AX, MDC1, 53BP1, *etc.*) that assemble into macromolecular foci surrounding DSBs to amplify damage signals and tether DSB ends for efficient repair [1]. Rif1 has been recently identified as a phospho-53BP1-

associating effector protein that plays a crucial role in CSR by suppressing DSB resection [14,15] (see details in following sections). Later studies have identified a series of Rif1 downstream effector proteins for resection inhibition including Rev7 [16,17], the Shieldin and CST complex [18–21] that are all required for efficient class switching.

Classical End-joining and Lymphocyte Development

c-NHEJ in mammalian cells is catalyzed by Ku70/Ku80 and XRCC4/DNA ligase IV (Lig4) complexes, and DNA-PKcs and Artemis in certain circumstances [4,10]. Briefly, this reaction is initiated by the binding of Ku to the broken DNA ends. A ring-shaped heterodimer complex that encircles the free ends, Ku recruits other components of the c-NHEJ machinery including DNA-PKcs, with which it comprises the DNA-PK holoenzyme, and the DNA ligase complex Lig4/XRCC4 [4,10]. Besides its role in activating Artemis that is absolutely required for hairpin end opening in V(D)J recombination, DNA-PKcs may also tether broken DNA ends that do not require further processing for efficient repair [22]. Additional c-NHEJ factors have been identified, including XLF, PAXX and ERCC6L2 [23]. c-NHEJ dominates the repair of programmed DSBs in V(D)J recombination and CSR. Mice deficient in these factors exhibit marked growth defect and immunodeficiency phenotypes, and cells deficient in these factors show significant radio-sensitivity and DSB repair defect to various degrees. V(D)J recombination is nearly completely abolished in the absence of either Ku, XRCC4 or Lig4 [10]. This constraint appears to be related to the ability of Rag proteins to limit alternative end-joining, as a Rag2 mutant that lost chromatin binding (Rag2-core) permits A-EJ mediated V(D)J recombination and causes genome instabilities [24,25]. XLF physically interacts with XRCC4 and stimulates the ligase activity of Lig4 [26,27]. However, it appears that XLF is largely dispensable for V(D)J recombination in developing mouse B and T cells [28], although mutations in human XLF that cause primary immunodeficiencies have been identified [27,29]. XLF has been shown to function redundantly with the DSB response kinase ATM and its downstream factors such as 53BP1 at least in part by suppressing end degradation [30–32]. PAXX has been identified as a c-NHEJ factor in recent years by structure similarity with XRCC4 and physical interaction with the Ku complex [33,34]. *In vitro*, PAXX promotes Ku-dependent DSB ligation and the assembly of c-NHEJ proteins on damaged chromatin. However, PAXX-deficient mice develop normal T and B cells, and exhibit embryonic lethality and apparent defect in V(D)J recombination when combined with XLF deficiency, indicating redundant roles for these two factors in c-NHEJ repair [35–38]. MRI is another XLF-like molecule identified recently that promotes c-NHEJ [39]. Mechanistically, MRI physically interacts with and promotes the retention of DDR and c-NHEJ proteins at the break site, and is required for c-NHEJ-mediated DSB repair in XLF-deficient lymphocytes. In addition, MRI-deficient mice are embryonic lethal when XLF is also absent, suggesting redundant roles for MRI and XLF in mediating c-NHEJ repair [39]. ERCC6L2 is another recently identified factor for optimal c-NHEJ [40,41]. ERCC6L2 physically interacts with c-NHEJ factors and are rapidly recruited to DNA damage site, and functions redundantly with XLF in mediating V(D)J recombination by c-NHEJ [40]. Taken together, multiple XRCC4/XLF paralogs perform redundant/overlapping roles in mediating c-NHEJ repair for V(D)J recombination during lymphocyte development [42].

Mounting evidence has clearly revealed that c-NHEJ is required for efficient CSR in mature B cells. Unlike c-NHEJ deficiency leading to nearly null V(D)J recombination, significant residual class switching still occurs in c-NHEJ-deficient mature B cells. XRCC4/Lig4, Ku70/80 or both-deleted mature mouse B cells have reduced CSR to all downstream isotypes at ~30%–50% of the corresponding wild-type levels, with concomitant unrepaired *IgH* and general DNA breaks [5,43,44], indicating that c-NHEJ promotes class switching and suppresses genome instability in mature B cells. Likewise, Lig4 mutations in human B cells greatly impair but do not completely abolish CSR [45]. DNA-PKcs is recruited to DSBs by Ku and in turn activates endo/exonuclease Artemis by phosphorylation to process complex ends before joining [4]. While DNA-PKcs is required for CSR to most isotypes other than IgG1 [46], Artemis plays a rather minor role in CSR, as Artemis-deficient B cells exhibit normal class switching and antibody production to most Ig isotypes [47,48], indicating that end processing of S region DSBs by Artemis is not a prerequisite for c-NHEJ in CSR. Deletion of XLF in mouse mature B cells shows substantial reduction in CSR and accumulation of *IgH* breaks [28], reflecting different roles for XLF in CSR and V(D)J recombination. On the contrary, MRI ablation only modestly affects class switching whereas PAXX is largely dispensable for CSR [49], likely suggesting functional redundancies of these XRCC4/XLF paralogs in mediating c-NHEJ in class switching as they do in V(D)J recombination. The precise roles for these factors in CSR need further dissection. ERCC6L2, however, plays an entirely different role in class switching, as ERCC6L2-deficient B cells show profound CSR defect and accumulation of inversional joins between Sμ and downstream S region DSBs that leads to nonproductive *IgH* rearrangement [40].

Alternative End-joining-mediated Class Switching in B Cells

Alternative end-joining was first reported in budding yeast with reporter assays to recover restriction enzyme-linearized plasmid in Ku80-deficient *S. cerevisiae* cells [50]. Similar c-NHEJ-independent joining was later discovered in other model organisms [51–54]. Junction profiles of Ku/Lig4-independent joins exhibit deletion of sequences around the DSBs and a strong bias towards MH, implicating the involvement of DSB resection in A-EJ. It is noteworthy that Ku-deficient or Ku/Lig4 double-deficient cells still contain substantial direct CSR joins [5], and there are also a significant portion of MH CSR junctions in wild-type B cells. Thus it appears that MH is neither a requirement, nor an exclusive defining factor for A-EJ. Rather, A-EJ shall be more accurately defined as any DSB end-joining occurring independent of classical NHEJ. Based on this definition, measuring end-joining efficiency in the absence of both target genes and c-NHEJ is needed to functionally define an A-EJ factor. To date, routine assays for end-joining may include radiation/drug sensitivity, nuclease (I-SceI or Cas9)-mediated reporter joining, V(D)J substrate recombination and CSR including S-S junction analysis, and cytogenetics experiments (*IgH* FISH, telomere FISH, *etc.*). With these assays, several genes uniquely required for A-EJ but not c-NHEJ have been identified. Functional studies of these factors indicate that they are involved in the following steps in A-EJ: (1) tethering broken ends; (2) end processing especially resection to generate 3′ ssDNA; (3) trimming off non-homologous flaps formed by MH annealing; (4) DNA fill-in synthesis; and (5) end ligation by DNA ligases other than Lig4. The fol-

lowing sections will introduce recent findings on the roles of these factors in A-EJ.

DSB end-tethering

A role for Ku in c-NHEJ is to hold broken ends for efficient repair. In the absence of Ku, other protein(s) must exist in place of Ku to tether DSBs for A-EJ repair. Parp1 has been proposed by multiple studies to serve this role. As a member of the poly(ADP-ribose) polymerase (PARP) family, Parp1 catalyzes the covalent attachment of poly(ADP-ribose) (PAR) on amino acid residues of target proteins with β-NAD+ as substrates [55]. Poly ADP-ribosylation of target proteins is involved in essential cellular processes, including transcription, replication, and DNA repair [55]. Parp1 has been shown to be activated by DNA breaks and serves as a DNA damage sensor [56]. Following laser microirradiation, Parp1 colocalizes with DDR kinases ATM and γ-H2AX, and is recruited to I-SceI-generated site-specific DSBs to promote the accumulation of MRN complex through physical interaction with Mre11 [57]. Biochemical experiments showed that Parp1 competes with Ku for DNA ends, albeit with lower affinity, and Ku efficiently counteracts the binding of Parp1 and MRN complex to damaged chromatin. In Ku or DNA Lig4-deficient cells, end-joining of EGFP reporter plasmids was significantly diminished upon Parp1 ablation [58]. In addition, Parp1 can recruit the XRCC1/Lig3 ligation complex to promote end-joining repair [59], and promote DNA synapsis in a dose-dependent manner, an activity that is independent of XRCC1/Lig3 [60]. Collectively, these studies suggest that Parp1 promotes A-EJ repair likely through DSB end-tethering and recruitment of downstream repair factors.

The potential role of Parp1 in A-EJ mediated CSR in activated B cells has been investigated. Pharmacological inhibition of Parp1 activities by a commercial small chemical surprisingly increased IgA switching on the mouse mature B cell line CH12F3, whereas this inhibitor, or the genetic deletion of Parp1 did not appear to affect the class switching efficiency to IgG in primary mouse B cells [61]. Sμ-Sγ3 junctions in Parp1-deficient sequences indeed display a shift towards less MH compared with WT cells. These observations led to a model that Parp1 mediates A-EJ in CSR by competing with c-NHEJ and promoting MH usage [61]. However, this study did not examine the CSR phenotype of B cells deficient in both Ku and Parp1 to definitively prove the A-EJ status of Parp1. It is also not clear whether the poly ADP-ribosylation activity of Parp1 plays any role in A-EJ-mediated CSR, and which downstream targets it modifies if it does.

DSB end resection

Sequencing remnant S-S junctions in c-NHEJ-deficient or DDR-deficient cells revealed frequent deletions and a strong bias to MH [6,12,62], implicating that S region DSBs in these cells are subject to nucleolytic end resection to expose 3′ ssDNA [2]. It has been well documented that 5′–3′ DSB end resection is required for homologous recombination (HR) and MMEJ in yeast and higher eukaryotes [2], and MMEJ and HR essentially share the same initial end resection mechanism [8]. DSB resection is initiated by the coordinated action of the DNA nuclease complex MRN and CtIP. In addition to endonuclease activity, the Mre11 protein in the MRN complex also possesses a 3′–5′ exonuclease activity, an opposite polarity to the ongoing resection. It turns out that Mre11 nicks DNA 3′ downstream to DSB with its endonuclease activity, and further degrades DNA using its exo-activity in a 3′-to-5′ orientation towards the break to expose 3′ ssDNA [63,64]. Human CtIP functions in resection initiation by stimulating Mre11's endonuclease activity [65,66], and CtIP phosphorylation at T859 is critical for its role in resection [67,68]. The identified exonuclease EXD2 has been shown to functionally interact with MRN to accelerate DSB resection and is required for efficient HR [69]. After the initial about hundred nucleotides DNA degradation, helicase BLM/WRN and endonuclease DNA2 switch on to carry out long-range resection up to tens of kilobases away from the break, and this activity appears redundant with exonuclease *Exo*1 [2].

The role for DSB resection in A-EJ has been a topic under extensive investigation. Depletion of Mre11 in wild-type cells reduces the use of MH, and Mre11 inhibition in XRCC4-deficient cells further suppresses end resection, decreases frequencies of joining adjacent I-SceI breaks in reporter systems, revealing specific roles for Mre11 in both classical and alternative NHEJ [70,71]. *Mre11*$^{+/-}$ B cells or cells carrying Mre11^{H129N}, the nuclease-dead mutant form of Mre11, both show severely impaired CSR. Analysis of S-S junction profile in the residual joining revealed that the overall patterns (direct vs MH) in Mre11 mutant cells are not significantly altered, consistent with the roles of Mre11 in both the c-NHEJ and A-EJ pathways [72]. Previous studies with high-throughput sequencing of S-S junctions revealed that Mre11 knock-down in wild-type B cells exhibits slightly but significantly increased MH usage while reducing CSR efficiency, further confirming Mre11's role in promoting c-NHEJ likely through activation of ATM-dependent DDR [73,74]. Mre11 knockdown in *Lig4*$^{-/-}$ B cells significantly further reduces class switching. Moreover, inhibiting either the exonuclease or the endonuclease activity of Mre11 by small chemicals renders severe CSR defect to *Lig4*$^{-/-}$ cells, implicating that both activities of Mre11 are required for A-EJ-mediated CSR [74]. In line with the functional interaction of Exd2 with Mre11 in resection initiation [69], Exd2 knockout in *Lig4*$^{-/-}$ cells but not wild-type cells conferred further CSR defect [74].

CtIP and MRN complex are required for several DNA repair pathways including HR and A-EJ. Silencing CtIP reduces joining frequency of I-SceI DSBs to a similar extent as Mre11 inhibition or Mre11/CtIP double-inhibition, indicating that Mre11 and CtIP are involved in the same pathway [70]. Previous studies with an I-SceI reporter system showed that A-EJ efficiency is significantly reduced in CtIP-depleted cells, while the absolute level of total-NHEJ was slightly increased in CtIP-depleted cells. Thus, CtIP promotes A-EJ, but is dispensable for the absolute levels of total-NHEJ [75,76]. It has also been shown that robust end-to-end chromosome fusions in *Lig4*$^{-/-}$ MEFs mediated by the A-EJ pathway is dependent upon CtIP [77]. In mouse ES cells, chromosomal translocations of I-SceI-mediated DSBs by A-EJ were significantly lower in CtIP-depleted cells than in control cells. Sequencing translocation junctions revealed significantly shorter MH and deletions in CtIP-depleted cells [78]. These studies support a role for CtIP-mediated resection in A-EJ and chromosomal translocations. However, the role of CtIP in CSR has been less clear [79–81]. Previous studies showed that CtIP knockdown in CH12F3 cells resulted in CSR deficiency with reduced MH length at the Sμ-Sα junctions [80]. The CSR defect in CtIP-deficient cells has been attributed to impaired proliferation or AID expression [80,81], although other possibility such as DSB end-bridging independent of resection initiation cannot be excluded [82]. It has been shown recently by high-throughput sequencing

that CtIP deletion in CH12 cells does not change the MH profile of the remaining junctions [74,81], implicating an Mre11-independent role of CtIP. Knocking down CtIP in Ku70-deficient, or *Lig4*$^{-/-}$ CH12 cells further reduced CSR, supporting an A-EJ role for CSR of CtIP in c-NHEJ-deficient cells [80]. Prior reports indicated that phosphorylation of CtIP at T859 by ATM or ATR kinase is essential for its role in resection initiation [67,68]. Treating CtIP knockdown with ATM inhibitors, or deletion of ATM in combination with CtIP knockdown in *Lig4*$^{-/-}$ B cells resulted in even a greater CSR defect than ATM inhibition or CtIP silencing alone [74]. While this observation is not necessarily conflicting with prior reports [83], suggesting that ATM might also activate other downstream targets essential for A-EJ other than CtIP.

The involvement of long-range resection in A-EJ-mediated CSR has also been investigated. Although BLM has been shown to be dispensable for resection initiation for MMEJ [8], high-throughput sequencing of S-S junctions and Cas9-mediated CSR assays demonstrate that BLM, together with its interaction partner Dna2, is required for CSR in *Lig4*$^{-/-}$ cells, and BLM knockout significantly diminishes CSR joins involving long DSB resection [74]. Surprisingly, the other resection factor Exo1, when only the resection but not mismatch repair-related function (essential for AID-induced DSB formation in activated B cells) is mutated, does not impact A-EJ at all [74]. Taken together, these recent findings demonstrated that both short-range and long-range resection play essential roles in mediating A-EJ repair in c-NHEJ-deficient cells.

Annealing of microhomologies

Following DSB resection in c-NHEJ-deficient cells, exposed ssDNA around broken ends is annealed to each other using embedded internal MH sequences for the ensuing DNA synthesis and ligation. Thus proteins mediating MH annealing serve as A-EJ factors by definition. It has been proposed that single-strand annealing protein Rad52 may perform such a role [84]. Rad52 is generally considered as a HR/SSA protein that mediates the displacement of ssDNA-bound RPA with Rad51 recombinase for homology invasion and homologous ssDNA annealing. However, mammalian Rad52 also binds directly to DNA ends, protects them from nuclease degradation, and promotes end-to-end interaction [85]. Zan *et al.* [84] showed that Rad52 is recruited to S region and competes with Ku for DSB binding in wild-type B cells. Rad52-deleted B cells show elevated CSR both *in vitro* and *in vivo* with reduced MH usage at S-S junctions, and ectopic expression of Rad52 in wild-type cells significantly inhibits CSR. In addition, Rad52 appears to promote intra-Sμ DSB joining that has a higher chance for MH paring. Importantly, knockdown of Ku86 in *Rad52*$^{-/-}$ B cells essentially ablates IgG switching [84]. This study implied that Rad52 could potentially regulate CSR in two scenario-specific modes: in wild-type cells it competes with Ku for S region DSB binding with a different synapsis configuration [84] that favors intra-S joining, which inhibits IgG-producing long-range Sμ-Sγ joining; in Ku-deficient cells, however, Rad52 mediates annealing of resection-generated Sμ and Sγ ssDNA for A-EJ CSR. How exactly the different functions of Rad52 are regulated in these scenarios is an interesting question for future investigation.

A recent study identified a protein called HMCES (5-*H*ydroxy*m*ethyl*c*ytosine Binding, *ES* Cell Specific) as a novel MH annealing factor for A-EJ [86]. HMCES-deficient B cells exhibit a mild CSR defect to IgA, IgG1 and IgE with reduced MH and elevated direct joins in S-S junctions. In addition, double-deficiency in HMCES and c-NHEJ factor Ku or Lig4, but not CtIP, nearly completely abolish CSR in CH12F3 cells. In support of a role in A-EJ, HMCES-deficient cells exhibit marked reduction in A-EJ efficiency in the EJ2-GFP reporter assay [86]. Mechanistically, HMCES can bind 3′ and 5′ ssDNA overhangs to facilitate their annealing for A-EJ, while at the same time protect ssDNA ends from excessive resection by Exo1 [49,86]. As both Rad52 and HMCES are crucial for A-EJ-mediated CSR in Ku-deficient cells [84], how these two proteins function in relation to each other in the context of A-EJ is of interest for further study.

It should be noted that MH in A-EJ can be as low as 1 deoxynucleotide (nt) in mammalian cells, whereas in yeast it usually requires longer homologous sequences [87,88]. In addition, the RPA complex binds to ssDNA ends to prevent spontaneous annealing and thus impede A-EJ in yeast [89]. The lower MH length requirement for stable base pairing in mammalian cells implicates additional proteins in synapsis or elongation of annealed MH. In this regard, the A-family DNA polymerase Polθ has recently been shown to possess the activity to anneal 3′ ssDNA overhangs with imbedded MH in addition to extending DNA templates (see details below).

Removal of nonhomologous flaps

Annealing of MH imbedded in resection-generated ssDNA between Sμ and downstream S regions produces 3′ terminal ssDNA flaps beyond the duplex region that must be removed prior to DNA synthesis. It is conceivable that such 3′-flap removal proteins would represent valid A-EJ factors. The structure-specific endonuclease XPF/ERCC1 complex specifically cleaves the junction between a single strand and duplex DNA where ssDNA moves away 5′ to 3′ from the duplex [90,91], and this polarity permits XPF/ERCC1 to take important parts in various DNA repair pathways, including nucleotide excision repair [91], inter-strand crosslink repair [92,93], and replication fork re-establishment [94]. Furthermore, XPF/ERCC1 has been implicated in telomere maintenance. Upon loss of telomere-repeat binding factor TRF2, telomeres are resected as single-ended DSBs to expose 3′ G-rich overhangs, and XPF/ERCC1 serves to cleave such overhang DNA to prevent telomeric recombination and shortening [95]. In addition, mammalian XPF/ERCC1 is essential for the repair of DSBs by homology-dependent gene conversion and SSA pathways, and is synergistic in IR sensitivity with Ku [96,97]. The homolog of XPF/ERCC1 in budding yeast, Rad1/Rad10 heterodimer, plays critical roles in DSB repair by single-strand annealing (SSA) by using its 3′-flap endonuclease activity [98] that highly resembles A-EJ in mammals and also requires interaction partner SLX4 [99,100]. A previous study using an EJ2-GFP reporter indicated that ERCC1 plays a mild role in A-EJ [75]. However, this study was carried out in cells proficient for c-NHEJ that had limited resection. Discrepancies in the role for XPF/ERCC1 in DSB repair during B cell class switching exist; reports of normal antibody production in XPF-deficient mouse B cells [101], in *Ercc1*$^{-/-}$ B cells [102], or reduced CSR to IgG and IgA in B cells from an independent *Ercc1*$^{-/-}$ mouse line have emerged [103]. A recent study investigated the role for XPF, ERCC1 and SLX4 in CSR in CH12F3 cells. Deficiency in any of these proteins in wild-type cells does not confer any CSR defect; instead, depletion of either XPF/ERCC1 or SLX4 results in a significant reduction in class switching in *Lig4*$^{-/-}$ or *53bp1*$^{-/-}$ cells [104]. On the other hand, complementing *Ercc1*$^{-/-}$*Lig4*$^{-/-}$ cells with an ERCC1 mutant that specifically loses 3′-

flap removal but not NER activity fails to rescue the switching phenotype. More importantly, high-throughput sequencing of residual Sμ-Sα junctions demonstrated joining to "long" resected Sα breaks are diminished in *Ercc1*$^{-/-}$*Lig4*$^{-/-}$ cells compared to that of *Lig4*$^{-/-}$ cells, confirming a role for XPF/ERCC1 in mediating A-EJ CSR through 3′ flap removal activity [104].

It is noteworthy that there are residual CSR and joining to long resected Sα DSBs in *Lig4*$^{-/-}$*Ercc1*$^{-/-}$ cells, suggesting functional redundancy with XPF/ERCC1 in flap removal that may stem from different sources. The Mus81-EME complex can be such a plausible candidate. Mus81 belongs to the XPF/Mus81 family of structure-specific endonuclease with specificity on double-Holliday junctions, stalled replication forks and 3′-flaps [91]. However, Arabidopsis thaliana Mus81 only plays a very minor role in SSA that absolutely requires 3′-flap removal [105]. It is thus of interest to test whether the Mus81-EME1/2 complexes indeed play any role in A-EJ-mediated CSR. Second, some DNA polymerases may use intrinsic proofreading 3′-to-5′ exonuclease activity to remove short flaps. Yeast Rad1/Rad10 has been shown to be critical for removing 3′-flaps longer than 30 nt in length, and XPF/ERCC1 binds to single-stranded overhang 15 nt or longer with maximal affinity [106,107]. Owing to dense AID targets in S regions, the exact location of AID-initiated DSBs and accurate length of resection are difficult to measure. S region DSBs undergoing short range resection, or MH annealing near the end of ssDNA can leave short or no 3′-flaps that are suitable substrates for proofreading DNA polymerases. In this regard, budding yeast Polδ and Pol4 (Polλ homolog) are required for MMEJ repair [88,108,109]. Additionally, mammalian translesion synthesis polymerase Polθ is capable of extending mismatched termini by endonucleolytic end-trimming of 3′-ends [110,111], and has recently been shown to be required for A-EJ and translocations [112–114] (see details below).

Several reports have shown that the 5′ flap endonuclease FEN1 plays a role in MMEJ using reporter assays and cell extracts [115,116]. It is speculated that FEN1 is responsible for cleaving off the 5' flaps generated by Polθ-mediated gap filling during MMEJ [117]. Mouse B cells with nuclease-dead FEN1, E160D, exhibit normal CSR and somatic hyper-mutation [118], precluding a role for FEN1's endonuclease activity in c-NHEJ. Another report suggested that FEN1, when recruited through interaction with UNG, acts as a BER factor to introduce mutation into *IgH* locus [119]. Given the staggering nature of clustered S region breaks, a portion of them may contain 5′ overhang that could be enlarged by the resection of adjacent breaks. The annealing of ssDNA containing such 5′-overhang produces 5′ flaps, and FEN1 could likely cleave such 5′-flaps before gap fill-in synthesis to facilitate A-EJ repair. As FEN1 is crucial for processing 5′ ends of Okazaki fragments during lagging strand replication and BER, two essential processes for CSR, proper separation-of-function mutants of FEN1 that distinguish these functionalities are required in order to dissect FEN1's potential role for A-EJ during class switching.

Fill-in DNA synthesis

Recent studies have identified several DNA polymerases including Polθ (encode by *Polq* gene) and Polδ as required for microhomology annealing and/or gap fill-in synthesis during MMEJ. Polθ was identified by sensitivity screening to interstrand cross-linking agents in Drosophila, and was associated with A-EJ during p-element transposition [120,121]. Polθ possesses polymerase activity that can effectively extend single-stranded DNA as well as duplex DNA with either protruding or mismatched 3′-OH termini [110,122]. Purified human Polθ protein executes MMEJ on DNA containing 3′ ssDNA overhangs with ≥2 bp of microhomology. Mechanistically, Polθ promotes annealing of MH sequences and then uses the opposing overhang as a template to extend the DNA, an action that further stabilizes the DNA synapse [113,123]. Interestingly, Polθ also harbors a robust end-trimming activity for nonhomologous overhangs that is intrinsic to its polymerase domain [111,124]. This endonucleolytic cleavage specific for the 3′-end allows a quick switch to its intrinsic DNA polymerase mode to extend the 3′-end at the microhomology annealing site. Besides the polymerase domain, Polθ also has an N-terminal helicase domain that promotes MH annealing [121,125], an activity that would likely stimulates A-EJ [123]. Polθ deficiency sensitizes mouse cells to DNA double-strand breaking agents such as etoposide and camptothecin. Moreover, Polθ promotes A-EJ and suppresses HR through physical interaction with Rad51 to expel RPA from ssDNA filaments; In this regard, Polθ inhibition confers synthetic lethality with HR-deficient cells [112]. Meanwhile, inhibition of Polθ inhibits MMEJ at dysfunctional telomeres [114]. Nonetheless, B cells from Polθ-defective mice exhibit overall normal CSR to different isotypes. Sequencing S-S junction in *Polθ*$^{-/-}$ B cells showed greatly diminished A-EJ-dependent insertions of > 1 bp at the CSR junctions in Polθ-deficient cells compared with that of wild-type cells, suggesting that Polθ is involved in A-EJ-mediated CSR by promoting templated nucleotide (T-nucleotide) insertion [84,126]. Taken together, these studies support a role for Polθ in promoting MH-mediated joining.

Two other DNA polymerases have been shown to play important roles in A-EJ, especially in mediating MH-mediated DSB repair and translocations. In budding yeast, Pol32 (components of replicating polymerase Polδ in eukaryotes) and Pol4 (related to Polλ in mammalian cells) promotes both MH-mediated DSB repair and chromosomal translocations as gap fill-in polymerases that may introduce T-nucleotide insertions into junctions [88,100,108]. The action mode of Pol4 in T-nucleotide insertion resembles Polθ in flies and vertebrates that involve initial MH annealing, followed by extension through error-prone polymerase activity, and ensuing dissociation of nascent DNA from its template for re-annealing to regions with secondary microhomologous sequences. In human cells, RNAi-mediated knock-down of POLD2, the accessory subunit of Polδ, reduces MH-mediated joining in the EJ5-GFP reporter assay, and additive Lig4- or 53BP1-knockout inhibits CRISPR/Cas9 breaks-mediated chromosomal translocations [127]. Together, these findings are consistent with prior reports that yeast Polδ can promote 3′ end processing, MH-mediated end-joining, and translocations [108,128]. The role of these polymerases in lymphocyte development, especially in c-NHEJ-deficient background, awaits more future investigations.

DNA ligases in A-EJ

There are three ATP-dependent DNA ligases, namely Lig1, Lig3 and Lig4 in vertebrates. Lig4 plays an exclusive role in c-NHEJ, while Lig1 is the major DNA replication ligase [129]. Therefore, it should be either Lig1 or Lig3 for ligation in A-EJ in the absence of Lig4. XRCC1 forms a stable complex with and stabilizes Lig3 [130,131]. Human and mouse Lig3 are present in both the nucleus and mitochondria [132], with the latter being essential for mitochondrial function and cell viability [133]. Previous biochemical experiments

with nuclear extracts or recombinant proteins in a plasmid-rejoining assay indicated that A-EJ requires the XRCC1/Lig3 complex [60,134]. Depleting Lig1 or Lig3 in human HTD114 cell nuclear extracts can significantly reduce MMEJ, and siRNA-mediated knockdown of Lig1 or Lig3 reduced the usage of MH [135], suggesting the redundant role for Lig1/Lig3 in A-EJ. However, it has been recently proposed that Lig3 may play a greater role in A-EJ than Lig1 does [136,137]. Kinetic analyses showed that Lig3 has greater affinity for DNA than Lig1 [138]. The study in chicken DT40 cells also implicated that Lig1 functions in A-EJ as a backup for Lig3 [139]. Knockdown of Lig1 did not change the use of MH and translocation formation by zinc finger endonucleases-generated DSBs, and nuclear Lig3 deficiency reduces translocation frequency [136]. These observations together suggest that while both Lig1 and Lig3 are involved in A-EJ, the Lig3-dependent pathway plays a major role in the repair of IR and Cas9-generated breaks.

Several studies have explored the potential roles for Lig1 and Lig3 in A-EJ-mediated CSR in B cells. Conditional inactivation of XRCC1 in wild-type or XRCC4-deficient primary B cells did not affect CSR or impact *IgH/c-myc* translocation formation. In addition, conditional knockdown of Lig3 in wild-type or Lig4-deficient primary B cells or CH12F3 B cell line did not affect A-EJ-mediated switching to IgA and *IgH/c-myc* translocation formation [140], indicating that XRCC1/Lig3 is not essential for A-EJ-mediated CSR. As Lig3 exists in non-essential nuclear form and essential mitochondrial form, to further definitively address the involvement of DNA ligases in A-EJ, two independent groups deleted either Lig1 or the nuclear form of Lig3 in wild-type and *Lig4*$^{-/-}$ CH12F3 cells and tested them for switching. Similar results were obtained that neither Lig1 nor nuclear Lig3 is indispensable for CSR in wild-type and *Lig4*$^{-/-}$ cells [141,142]. Taken together, these studies strongly demonstrate that Lig1 and nuclear Lig3 are functionally interchangeable to support robust A-EJ-mediated class switching.

A-EJ in the Context of V(D)J Recombination

It was initially believed that early B cell development does not allow alternative end-joining, as V(D)J recombination is nearly completely nonexistent in XRCC4-, Lig4- or DNA-PKcs-deficient mice [10], and *Ku70*$^{-/-}$ mice can display a severely impaired but also leaky SCID phenotype [143]. An early study of *IgH-c-myc* translocations in pro-B cell lymphomas recovered from XRCC4- or Lig4-deficient mice that also lack p53 discovered junctions with characteristics of end-joining and MH usage [144], indicating that translocation-prone A-EJ can occur in c-NHEJ-deficient B cells during V(D)J recombination. A recent report revealed with high-throughput sequencing that Ku proteins suppress A-EJ-mediated V(D)J recombination in G1-arrested *Lig4*$^{-/-}$ *v-Abl* pro-B cells. Knockout of *Ku70* in *Lig4*$^{-/-}$ cells rescues V(D)J recombination level that is comparable to Ku70-deficient cells, as well as A-EJ repair of DSBs generated by other engineered nucleases [145]. This study also nicely explains the differential impacts of Ku and XRCC4/Lig4 on the early lymphocytes and embryonic development [145]. Another recent report implicated that Polθ promotes A-EJ for V(D)J recombination in XRCC4-deficient pro-B cells that undergo extensive resection in S/G2 phase of the cell cycle. Such A-EJ generates products with long sequence deletion and MH usage, and chromosomal translocations [146].

It has been shown that RAG proteins strongly suppress A-EJ during V(D)J recombination, as a Rag2 C-terminal truncation mutation (termed Rag2-core) allows substantial level of A-EJ of plasmid V(D)J recombination in c-NHEJ-deficient cells and in developing lymphocytes [24,147]. The repair products in Rag2-core cells show a marked preference to deletion and MH usage in both coding and signal joints. This phenomenon was also observed in wild-type cells expressing the Rag2-core mutant [24]. These data help to explain previously confusing observations that Rag2-core knock-in mice bear excessive deletions and short sequence microhomologies in the coding and signal joints in addition to the immunodeficiency phenotype [148,149]. Mechanistically, Rag2-core destabilizes the RAG post-cleavage complex to allow translocation-like A-EJ-mediated V(D)J recombination [25]. Suppressing A-EJ by RAG benefits the production of a diverse immune receptor repertoire, as frequent deletions generated by A-EJ in Rag-core-harboring cells would disrupt the coding sequence of antibody genes, and more deleteriously, introduce oncogenic chromosomal translocations. The substantial amount of mature T cells in the thymus and spleen of Rag2-core mice indicate that similar A-EJ pathways can also operate in T cells. A later study revealed that an acidic hinge region within the C-terminal of Rag2 protein permits A-EJ-mediated V(D)J recombination and translocations [150]. Taken together, these studies suggest that RAG recombinases efficiently suppress A-EJ events during V(D)J recombination that would otherwise generate rare aberrant products or chromosomal translocations that are selected against by p53-dependent mechanisms. It is of interest to further dissect the molecular components that promote A-EJ in Rag2-core-bearing mice and lymphocytes.

A-EJ and Oncogenic Chromosomal Translocations

It has been well documented that in mice deficiency in c-NHEJ factors Ku, Xrcc4, or Lig4, significantly increases chromosomal translocations in Pro-B [144], mature B [44,151,152] and ES cells [153]. These translocations predominantly harbor short MHs, suggesting that they are generated by MH-mediated end-joining. This notion has been confirmed by a study with high-throughput sequencing of genome-wide translocations, which showed that translocations joining *c-myc* DSBs to genome-wide DSBs in 53BP1-deficient cells, and to a lesser extent in ATM- or Rif1-deficient cells, display similarly MH-biased profile [6]. As extensive resection of S region breaks and *c-myc* breaks have been observed in these cells [12], these data suggest that DSB resection promotes chromosomal translocations through potentiating A-EJ, although other possibilities such as loss of DSB tethering and chromatin synapsis cannot be excluded [12]. In line with a role for DSB resection in promoting translocations, inhibition of CtIP [78] greatly diminishes I-SceI-induced chromosomal translocations in wild-type and Ku70-deficient mouse ES cells.

Recent studies have identified additional factors that regulate chromosomal translocations via the A-EJ/MH pathways. Parp3, a member of the PARP family of enzymes, has recently been shown to promote I-SceI-induced translocations in wild-type and *Ku70*$^{-/-}$ mouse ES cells. Although Parp3 deletion appears not to impact the MH profile in the remaining translocation junctions, it does reduce the average length of deletion [154]. Two possible explanations have been proposed for this phenomenon: first, *Parp3*$^{-/-}$ cells have less deposition of RPA at DSBs, indicating that Parp3 promotes DSB processing to generate ssDNA. Second, Parp3 may negatively regulate the binding of Ku to DSBs [154]. But as the Parp3 deletion also reduces translocations in *Ku70*$^{-/-}$ cells, there exist other mechan-

isms for Parp3-dependent promotion of translocations. Moreover, an earlier study reported conflicting results that Parp3 negatively regulates CSR without affecting *c-myc-IgH* translocations [155]. Although this discrepancy has been attributed to difference in mouse genetic background [154], the exact roles of Parp3 in regulating CSR and chromosomal translocations need more clarification.

A recent study showed that XPF/ERCC1 endonuclease complex promotes *c-myc-IgH* translocations and does so only in Lig4-deficient but not in wild-type B cells, supporting an A-EJ-specific role for XPF/ERCC1 in translocation [104]. As XPF/ERCC1 promotes A-EJ-mediated CSR via the intrinsic 3′ flap removal activity following MH annealing, it is conceivable that XPF/ERCC1 also facilitates chromosomal translocations by flap removal to allow subsequent DNA synthesis and ligation. It is of interest to examine whether other enzymes potentially involved in flap removal play any role in promoting A-EJ-mediated translocation.

The discovery of the role of Polθ in translocation regulation has drawn much attention. Polθ appears to promote Cas9-induced translocations and nucleotide insertions at the junctions, and Polθ knockdown in MEFs also inhibits MMEJ-mediated fusion of dysfunctional telomeres [114]. Co-localization of Polθ with γ-H2AX is decreased after knockdown of *PARP1* via siRNA or chemical inhibitors, suggesting that PARP1 facilitates the recruitment of Polθ to DSBs [114]. A later study revealed that both the polymerase and helicase domains of Polθ are required for Cas9-mediated chromosomal translocations in mouse ES cells. *In vitro* experiments showed that Polθ helicase activity facilitates the removal of RPA from resected DSBs, enabling subsequent MH annealing and joining by A-EJ [156]. However, conflicting results over the role of Polθ in translocation have been reported. In activated spleen *Polθ*$^{-/-}$ B cells, the frequency of *c-myc-IgH* translocation was 4-fold higher than the corresponding wild-type B cells [126]. Polθ knockout in *Ku70*$^{-/-}$ but not in wild-type mouse MEFs also further increases Cas9-induced translocations [125]. It is likely that differences in the type or feature of DSB ends and the primary sequence and/or the genome location of the breaks would be attributable to these discrepancies, as the frequency of chromosomal translocation is proposed to be positively correlated with the probability of the synapsis frequency of the two DSBs [1]. Nonetheless, the exact role of Polθ in chromosomal translocation needs further clarification.

Lastly, Lig3 has been shown to be a positive regulator of chromosomal translocation, in line with its role in A-EJ. Frequency of translocations induced by zinc finger nuclease in nuclear Lig3-depleted mouse ES cells is about half-reduced comparing to wild-type cells, whereas a Lig4 deletion increases translocation frequency by 2–3 folds. Lig1 can serve as a backup ligase to mediate these translocations [136]. Consistently, deletion of nuclear Lig3 significantly reduces but not completely abolishes Cas9-induced translocations in *Lig4*$^{-/-}$ CH12 cells. Interestingly, the junctions in the remaining translocations utilize even more MH than that of *Lig4*$^{-/-}$ cells, reflecting a unique preference for longer MH by Lig1 in mediating translocation [142]. Collectively, these results clearly indicate that A-EJ by mouse Lig3 and Lig1 promotes chromosomal translocations.

It is noteworthy that although mounting evidence has clearly demonstrated that A-EJ but not c-NHEJ promotes chromosomal translocation in mouse ES cells, MEFs and B cells, a recent study indicated that in human cells XRCC4/Lig4 but not Lig3 is primarily responsible for chromosomal translocations induced by engineered nucleases, including ZFNs, TALENs and Cas9, with the residual junctions showing greatly increased large deletions and MH usage [157]. A similar observation was made with hypomorphic Lig4 mutations identified from human patients [157]. Thus it appears that human and mouse cells utilize very different mechanisms to mediate translocations that can be likely attributed to the differential requirement for c-NHEJ proteins such as DNA-PKcs and Ku in humans and rodents [157]. As the residual translocations in XRCC4/Lig4-deficient human cells still display features of A-EJ with significantly increased deletions and MH usage, it is likely that reduced translocations in these cells result from refractoriness to initiate or undergo DSB resection that is required for A-EJ.

Conclusions and Perspectives

Recent years have witnessed remarkable progress in the understanding of A-EJ not simply as a backup pathway(s) to c-NHEJ, but a pathway(s) that can potentially compete with c-NHEJ for DSB repair. A-EJ is distinct from c-NHEJ in both participating components and repair kinetics, and shares with HR early steps of DSB resection (Figure 3). The discoveries of specific factors in the subsequent ssDNA annealing, nonhomologous flap removal, gap synthesis and ligation make A-EJ unique in a way prone to oncogenic chromosomal translocations. Thus, studying the molecular mechanisms of A-EJ will potentially benefit the identification of cancer therapeutic drug targets. To further elucidate A-EJ mechanisms, future studies should focus on the unsolved questions including: (1) the relative contributions of c-NHEJ versus A-EJ in wild-type cells; (2) whether A-EJ represents a single pathway or multiple pathways; (3) how the cells choose DSB repair pathway: A-EJ versus c-NHEJ or HR? (4) the cell cycle regulation of A-EJ; (5) why human and murine cells adopt seemingly different mechanisms for chromosomal translocations; and (6) the contribution of individual A-EJ factors for lymphocyte development and malignancies, and many other exciting ones.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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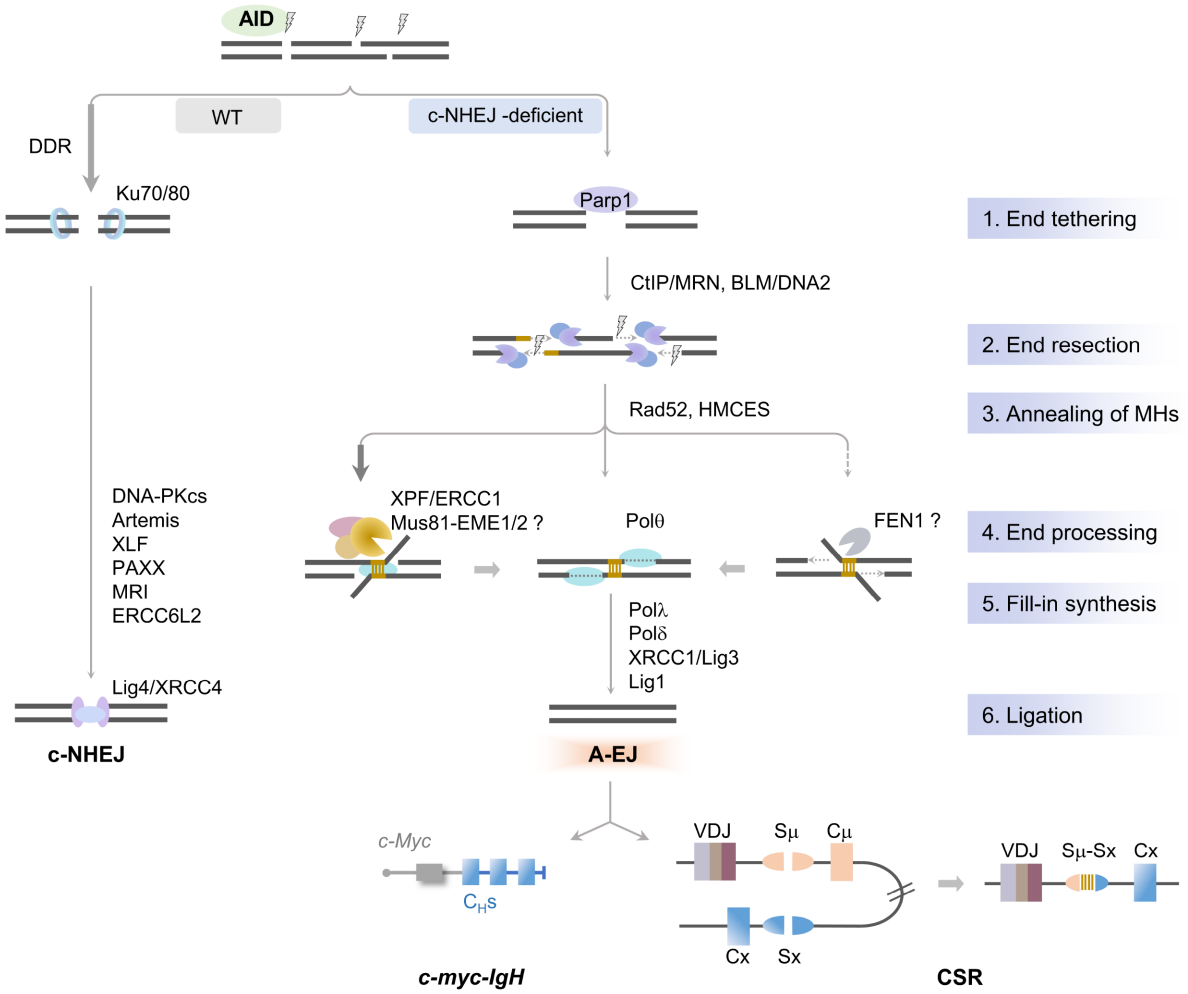

Figure 3. Summary of c-NHEJ and A-EJ during CSR and other DSB repairs In wild-type cells, c-NHEJ dominates DSB repair. DSB response kinase ATM and downstream factors such as 53BP1 enforce repair by c-NHEJ largely by end-tethering and preventing DSB end resection. Ku70/80 complex initially binds to DSBs to recruit DNA-PKcs/Artemis (in case complex end processing is needed) and XRCC4/Lig4 for ligation. XRCC4 paralogs (XLF, PAXX, *etc.*) play redundant roles possibly in end-tethering for efficient c-NHEJ. In cells deficient in c-NHEJ and thus with deprotected ends, PARP1 functions to hold DSBs in proximity and recruits other downstream processing and ligation proteins like Mre11 and XRCC1/Lig3. End resection by both the short-range and long-range resection machineries generates 3′ and potentially 5′ ssDNA (in case of AID-induced breaks) that anneal with each other through imbedded MH under the help of Rad52 and HMCES proteins. Polθ also participates in MH annealing and ensuing repair synthesis by endonucleolytic trimming-off of unmatched 3′-termini. The removal of nonhomologous flaps is mainly carried out by the structure-specific endonuclease XPF/ERCC1, likely redundantly by the Mus81-EME1/2 complex or FEN1. DNA polymerase Polδ, Polλ and Polθ function in gap fill-in synthesis, while both Lig1 and Lig3 are functional in the last ligation step. As a result, A-EJ in mouse B cells and other cells promotes the deletion of sequences, utilization of MH and chromosomal translocations.

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