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Alternative end-joining pathway(s): Bricolage at DNA breaks

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ABSTRACT

To cope with DNA double strand break (DSB) genotoxicity, cells have evolved two main repair pathways: homologous recombination which uses homologous DNA sequences as repair templates, and non-homologous Ku-dependent end-joining involving direct sealing of DSB ends by DNA ligase IV (Lig4). During the last two decades a third player most commonly named alternative end-joining (A-EJ) has emerged, which is defined as any Ku- or Lig4-independent end-joining process. A-EJ increasingly appears as a highly error-prone bricolage on DSBs and despite expanding exploration, it still escapes full characterization. In the present review, we discuss the mechanism and regulation of A-EJ as well as its biological relevance under physiological and pathological situations, with a particular emphasis on chromosomal instability and cancer. Whether or not it is a genuine DSB repair pathway, A-EJ is emerging as an important cellular process and understanding A-EJ will certainly be a major challenge for the coming years.

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1. Introduction

DNA double strand breaks (DSBs) are the most deleterious lesions inflicted on the genome. Discontinuity on both DNA strands may prove lethal for the cell if left unrepaired, or lead to chromosome aberrations and promote tumor development when misrepaired [1,2].

DSBs can arise from endogenous sources, mainly corresponding to accidental events like replication fork collapse following stalling or arrest at DNA damage or telomere deprotection [3]. More specialized mechanisms of DSB formation also exist that rely on development-associated programmed processes like meiosis during gametogenesis [4], or V(D)J recombination [5] and class-switch recombination (CSR) [6] which facilitate the rearrangements of antigen receptor genes in lymphogenesis. Aside from these endogenous sources, DSBs are also produced by environmental or medical sources of clastogenic injuries such as ionizing radiation (IR), radiomimetic chemicals or topoisomerase inhibitors [3,7].

Cells have evolved two main repair pathways to cope with DSB genotoxicity: homologous recombination (HR) which uses homologous DNA sequences as repair templates [8–10], and non-homologous end-joining (NHEJ) involving direct sealing of DSB ends [3,7,11,12].

In addition to the extensively studied predominant NHEJ pathway, an alternative end-joining mode (A-EJ) has emerged during the last two decades. Here, we review its mechanism and compare arguments suggesting that A-EJ relies on a single pathway, on various (sub)pathways or on no defined pathway. In addition, we report mounting evidence that although A-EJ may have little physiological relevance in normal cells due to several concurrent locks, A-EJ may particularly contribute to cancer through promotion of genetic instability and chromosomal translocation.

2. Historical overview and definition of A-EJ

Over the past two decades, the dominant NHEJ pathway has been thoroughly investigated and its genetic and mechanistic bases have been largely clarified (for reviews, see [3,7,11–13]). Briefly, the reaction is initiated by the binding of the Ku complex at each DNA end. Ku is a ring-shaped heterodimer composed of two subunits (Ku70 and Ku80) able to encircle the free ends. Once bound, Ku recruits the remaining components of the reaction including the catalytic subunit of the DNA-dependent proteine

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kinase (DNA-PKcs). Together these form an active serine/threonine DNA-PK holoenzyme belonging to the phosphatidylinositol 3-kinase-related kinases (PIKKs) family. Besides its essential catalytic function, DNA-PK has a major role in maintaining both DNA ends in close proximity, although recent findings have indicated a substantial role of the ligase complex in stabilizing this synapsis [14]. Both the DNA-PK and the ligase complexes are important for DNA end processing which is frequently required to make ends ligatable [15]. One of the processing activities is carried out by the structure-specific endonuclease Artemis, a DNA-PKcs partner which is also involved in hairpin opening during V(D)J recombination (see below, section 4). The ligation complex is composed of DNA ligase IV (Lig4), a homodimer of XRCC4 (X-Ray repair cross-complementing protein 4) which is indispensable for Lig4 stability, and the more recently identified Cernunnos homodimer (also known as XRCC4-like factor, henceforth referred to as Cer-XLF) whose exact function remains unclear. The Lig4 complex has no known function aside from its essential role in NHEJ, whereas components of the DNA-PK complex, especially Ku70/Ku80, have been implicated in multiple important cellular functions such as telomere maintenance [16], replication [17], transcription [18] or apoptosis [19].

The major role of NHEJ in response to IR-induced DSBs or during the V(D)J recombination is underlined by the cellular radiosensitivity (RS) and the severe combined immunodeficiency (SCID) phenotype routinely observed when one of the corresponding genes is mutated, whether in animal models or in human patients with hereditary RS-SCID syndromes [20,21].

Although NHEJ plays a critical role in DSB repair, a residual end-joining activity was reported in yeast mutated for Ku80, Ku70 or Lig4 [22,23]. The resulting repair products exhibited deletions and were strikingly characterized by a strong dependence on short homologous sequences at the junctions [22]. Similar NHEJ-independent end joining activities were also found in mycobacteria [24], *Arabidopsis* [25], *Caenorhabditis elegans* [26], *Xenopus* [27], chicken [28], as well as in rodent [29–32] and human cells [32–34]. Furthermore, when analyzed, the junctions consistently exhibited a greater use of microhomology (MH), compared to NHEJ. This novel universal alternative end-joining mode was susbequently termed MH-mediated end-joining (MMEJ), as opposed to the *canonical* or *classical* NHEJ, hereinafter called C-NHEJ. Although MMEJ resembles single-strand annealing (SSA), the MMEJ mechanism differs by being independent of Rad52 [35,36] and by using significantly shorter direct repeats [37]. Although the C-NHEJ-independent end-joining pathway is biased toward an increased use of MH, direct end joining and MH usage are not exclusive attributes of C-NHEJ and alternative end-joining pathway, respectively [3,38]. Moreover, apparently direct joints may yet arise from a MMEJ process using occult MH through non-templated nucleotide insertion by a TdT-like polymerase activity of polμ [39] or templated insertion by polθ [40,41]. Consequently, it seems prefereable to use the generic name alternative end-joining (A-EJ) instead of the restrictive term MMEJ and to define A-EJ as any Ku- or Lig4-independent end-joining process [42].

3. Molecular mechanism of A-EJ

3.1. A-EJ tools

A-EJ investigations have benefited from the development of a large number of assays that have been set up to study DSB repair by either NHEJ or HR pathways. Historically, *in vitro* end-joining assays as reviewed in Pastwa and coll. [43] first established important features of the NHEJ mechanism. The technique is based on incubation of linearized plasmid DNA or oligonuclotides bearing or not modified ends with cell extracts or purified proteins. These *in vitro* assays brought early insights into the mechanism of A-EJ [30,34,44–50]. Notably, low Mg^{2+} concentration were found to favor DNA-PKcs-dependent end-joining activity [51], whereas high Mg^{2+} concentration (10 mM) facilitated DNA-PK-independent reaction [34]. In addition, A-EJ preferred high DNA ends/protein molar ratios [44,52] or was favored by volume excluders like PEG [53], possibly indicating a weak intrinsic synapsis activity at DNA ends.

More recently, *in vivo* end-joining assays developed to study NHEJ have also contributed to establish features of A-EJ (Table 1). They use transient transfection of linearized reporter substrates followed by plasmid rescue, or cutting of intrachromosomal GFP-based reporter substrates by the rare endonuclease I-SceI [54]. End-joining is monitored by restoration of reporter gene expression combined with PCR amplification and DNA sequencing of the junctions. A drawback of transient transfection assays may be that significant differences in MH usage and/or end-joining efficiency are obtained depending on the transfection method employed [32,55]. Limitations of intrachromosomal assays concern first, the low frequency of double I-SceI cut at a single locus and second, underestimation of accurate NHEJ efficiency because iterative I-SceI cutting tends to select inaccurate repair.

Assessing repair of endogenous DSB generated during the two physiological processes of V(D)J and class-switch recombination (CSR) that relies on end-joining has also been useful to characterize A-EJ under conditions of C-NHEJ deficiency (Table 1). Although repair is measured on endogenous substrates, conclusions from these assays may not be entirely transposable to repair of any DSB. V(D)J recombination breaks are preferentially constrained to C-NHEJ and repetitive context sequence of CSR breaks favors MH usage. Finally, we have used a cellular fractionation protocol to study A-EJ in native chromatin. After treatment with a strong DSB inducer followed by western-blotting or immunostaining, recruitment of A-EJ proteins to damaged chromatin can be studied in cells engineered for Ku depletion [56].

3.2. A-EJ players

Based on the C-NHEJ mechanism, the A-EJ reaction likely relies on at least three main steps (Fig. 1). First, the two DNA ends must be recognized and held together. Second, although sometimes dispensable, most of DNA ends require processing to make them ligatable. Several enzymatic activities may participate in this step such as various types of nucleases, dRP lysases, kinases, phosphatases, helicases and polymerases. Third, the final step requires a DNA ligase and according to the proposed definition of A-EJ, this step in mammals should rely on DNA ligase III (Lig3) and/or DNA ligase I (Lig1).

In the next sections, we review the protein components potentially involved in the recognition/synapsis, processing and ligation steps of A-EJ.

3.2.1. End recognition and tethering of DSB ends

Several reports have established a role for poly(ADP-ribose) polymerase 1 (PARP1) in early steps of A-EJ. PARP1 is a sensor of DNA damage that binds to single strand breaks (SSBs) and DSBs, gets activated and catalyzes the poly(ADP-ribosyl)ation of proteins at DNA damage sites (reviewed in [57], [58]). The well documented role of PARP1 in SSB repair is to recruit factors including the ligation complex XRCC1/Lig3 to promote repair *via* DNA end processing and ligation [59]. PARP1 also participates in the initial accumulation of the MRE11/RAD50/NBS1 (MRN) complex to DSBs [60].

Concomitantly to the finding that PARP inhibitors increase the sensitivity of DNA-PK-deficient cells to radiomimetic-induced DSBs [45], biochemical experiments [45,48,49] and plasmid assays in Ku-deficient cells [61] substantiated the involvement of PARP1 in a non

Table 1
In vivo assays to explore A-EJ.

DSB location	Repair event	Origin of DSBs	Significant References
Transfected vector	DSBR	Restriction enzyme	[22,31,32,61,78,109,288]
	V(D)J	RAG	[32,132,139,289,290]
Integrated cassette	DSBR	I-SceI	[35,72,73,83,110,111,118,180,246,291–297]
	V(D)J	RAG	[70,137,138,140,159,244,257,298,299]
	CSR	I-SceI	[185]
	Translocation	I-SceI, RAG ± I-SceI, AID, Cre	[81,180,243–245,264,300–303]
Genomic DNA	V(D)J	RAG	[133,134,136,261,304]
	CSR	AID	[62,71,82,95,100,116,118,149–152,154,160,161,163,168,169,171,174,180,182,184,185,264,305–307]
	Translocation	IR, etoposide, Zn-finger nucleases	[94,240,245,303,308]
		RAG, AID, spontaneous	[62,95,100,152,154,162,163,171,240,261,269,302,309,310]

C-NHEJ mode of DSB repair, possibly through both tethering of DSB ends and protein scaffolding activities [45].

In the context of AID-induced DNA breaks generated during CSR, switch junctions in PARP1-deficient B cells are biased towards an absence of MH, indicating that PARP1 facilitates repair through A-EJ [62]. The fusion of deprotected telomeres in Ku-deficient cells is significantly reduced upon repression of PARP1 with an shRNA or treatment with a PARP inhibitor [63]. In Ku-deficient CHO cells, siRNA-mediated PARP1 depletion result in a severe defect in end-joining of chromosomal I-SceI DSB indicating that PARP1 may promote chromosomal A-EJ specifically in the absence of Ku [63]. A direct competition between PARP and Ku for binding to DNA ends [61] together with the finding that Ku inhibits PARP1 recruitment, poly(ADP-ribose) synthesis and generation of single-stranded DNA [56] suggest that suppression of A-EJ is mainly attributed to the presence of Ku at DNA ends (see below, section 4). Recently, a role of PARP in plant A-EJ was also confirmed as PARP mutants displayed less A-EJ products analyzed in an *in vitro* end-joining assay in *Arabidopsis* [64].

In addition to its possible role in A-EJ through tethering of DNA ends as supported by structural studies [65], PARP1 may also serve in this process as a platform for directly or indirectly recruiting factors such as XRCC1, Lig3, PNK [33,45,48] and MRN [56]. The latter may also contribute to tethering of DSB ends in A-EJ through its bridging function [66].

3.2.2. DSB processing

3.2.2.1. End resection. A current model is that, when necessary, A-EJ uses DNA resection to reveal single-stranded MHs that have to anneal before joining, therefore requiring nucleolytic DNA end processing [67]. As established for HR, the process of end resection comprises two steps: first, relative short stretches of ssDNA are produced by the combined action of MRN and C-terminal binding protein interacting protein (CtIP); then the EXO1 exonuclease or the Bloom (BLM) (Sgs1 in *S. cerevisiae*)/Dna2 endonuclease complex perform a more extensive resection (for review, [68]).

In *S. cerevisiae*, through the use of HO endonuclease to create DSBs, it was shown that Mre11 and Rad50 are required for MMEJ [36,69].

In mammals, by using a mutated RAG2 protein that permits A-EJ-mediated repair of V(D)J coding-joint formation, Roth's group revealed a role for NBS1 in V(D)J coding-joint formation in the absence of Artemis or DNA-PKcs [70]. In both human and murine cell lines the role of MRE11 in NHEJ has been assessed by studying the repair of I-SceI endonuclease-induced DSBs [71–73]. Reduction of MRE11 protein level or chemical inhibition of MRE11

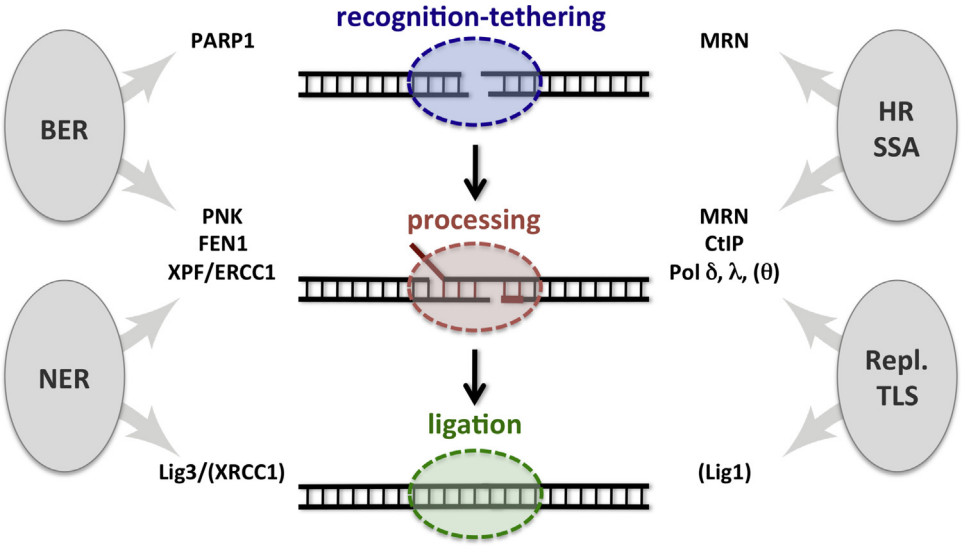


Fig. 1. A "catch-all" model for A-EJ. The three main steps of the reaction are depicted as well as the corresponding protein factors involved. Brackets signal minor or equivocal A-EJ factors. See the text for details (section 3.2). On both sides are indicated additional DNA transaction/repair pathways to which also belong these factors. BER, base excision repair; NER, nucleotide excision repair; HR, homologous recombination; SSA, single-strand annealing; Repl, replication; TLS, translesion synthesis.

decreased chromosomal end-joining in wild-type as well as XRCC4-deficient backgrounds, potentially implicating MRE11 in both C-NHEJ and A-EJ [72,73]. In this context, the MRN complex and the MRN-interacting CtIP probably work together, since depletion of NBS1 [74], RAD50 [72] or CtIP [72,73] in wild-type or XRCC4-defective cells also leads to a similar decrease in overall end joining.

Another study provided new insights into the mechanism of MRE11 during end-joining in human embryonic kidney cells containing a chromosomally integrated NHEJ substrate [75]. Silencing of MRE11 with siRNA leads to a significant reduction in MH-mediated deletional end joining compared with siRNA control cells. The frequency of A-EJ was significantly suppressed by siRNA-mediated MRE11 silencing whereas the frequency of C-NHEJ was not changed [75].

CtIP is required for DSB resection and participates, in cooperation with the MRN complex, in several DNA repair pathways including HR and A-EJ [35,76–78]. Short-interfering-RNA-mediated knockdown of CtIP in HEK293 cells [35] and knockout of CtIP in DT40 cells [78] result in a significant reduction in A-EJ. These data establish a role for CtIP in A-EJ thereby explaining the defects in overall DSB repair observed in CtIP mutants cells during G1 in response to X-ray damage [78]. Moreover, in the absence of H2AX, CtIP promotes hairpin opening and DNA end resection of RAG-mediated DSBs in G1-phase lymphocytes [79,80]. CtIP has also been implicated in the A-EJ-mediated formation of translocations originating from I-SceI-induced DSB in mouse embryonic stem cells [81]. Furthermore, in the physiological context of CSR, knockdown of CtIP expression in the mouse B cell line CH12F3 results in a reduction of CSR along with a significant decrease of the average MH length at Sμ-Sα junctions, thereby providing evidence for the requirement of CtIP for MH-directed A-EJ during CSR; additional data in this study demonstrated that CtIP is also involved in MH end-joining in Ku70-deficient cells [82]. Truong and coll. used an MMEJ and HR competition repair substrate in human cells and demonstrated that MRN and CtIP are required for the initial short-range end resection to promote MMEJ and that BLM and EXO1 are needed for extended end resection and HR [83]. Additionally, CtIP also supports resection associated with A-EJ dependent telomere fusions in various models [63,84].

3.2.2.2. Polymerase, flap endonuclease, helicase and polynucleotide kinase. In yeast, the translesion DNA polymerases Polη (Rad30 or polH) and Polζ (Rev3/Rev7), Pol4 (most homologous to Polμ and Polλ of the X family in mammals) and also the processive Polδ, through its accessory protein Pol32, have been proposed to participate in A-EJ [69,85]. Lee and Lee, using *in vivo* end-joining assays to repair HO-induced DSBs, proposed that Polδ, Polη (Rad30) and Polζ (Rev3) may contribute to MH-mediated end joining by extending synthesis beyond initial gap fill-in synthesis by Pol4 [69]. Using a repair assay that allows discrimination between C-NHEJ and A-EJ events based on the sensitivity to hygromycin, more recent studies in yeast show that deletion of Pol32 also severely reduces MH-mediated repair [86]. The role of Pol32 in MH-mediated repair is unlikely to recruit translesion polymerases because deletion of Polζ (Rev3) and/or Polη (Rad30) does not impact MH-mediated repair, but rather stabilizes the annealing intermediate between single-strand DNA to allow Polδ to extend the annealed homologous sequences and complete the repair process [86].

The mammalian ortholog of *S. cerevisiae* Pol4 is Polλ, a X family member of repair polymerases. Its structural and biochemical features make mammalian Polλ a likely candidate for the synthesis step in A-EJ, which involves unstable primer-template junctions resulting from annealing of 3′-ssDNA overhangs at MH regions. Polλ preferentially fills gaps with ends bearing partial complementary overhangs [39]. Indeed, in a minimal *in vitro* system, Polλ and Lig1 but not Polβ are able to carry out MH-mediated end joining of broken DNA ends with terminal MH [87].

In *D. melanogaster* which lacks PolX members, Polθ (PolQ) participates in alternative end-joining in response to P element-induced breaks or ISce-I mediated DSBs that operate independently of both Rad51-mediated HR and Lig4-dependent C-NHEJ. Genetic analysis provide support for distinct roles of N-terminal helicase-like and C-terminal polymerase domains in a model where both activities cooperate to generate single-stranded MH sequences used in A-EJ [40]. The role of Polθ in A-EJ in other organisms remains to be explored.

Yeast Rad1 and Rad10 proteins form a stable endonuclease complex that is required for several DNA repair pathways including nucleotide excision repair (NER) end HR. Genetic observations suggested that Rad1 and Rad10 may be needed for MMEJ by removing the 3′-flap DNA that forms upon annealing of MH. Indeed, deletion of both yeast Rad1 and Ku70 leads to a synergistic reduction in survival after induction of DSB by endonuclease HO [36]. These findings were supported by Villarreal and coll. showing that deletion of Rad1 reduced MH-mediated repair frequency [86]. Rad1-Rad10 activity in DSB repair are conserved in mammals, since the mammalian ortholog ERCC1-XPF also contributes to DSB repair in a Ku80-independent manner characteristic of A-EJ [88].

Despite its 5′ endo/exonuclease activity, Flap endonuclease-1 (FEN1) protein has also been proposed as a candidate in A-EJ. FEN-1 was detected in a partially purified fraction from *Xenopus* egg extracts devoid of Ku70/80 and DNA-PK and that contained an error-prone NHEJ activity that created deletion at patches of MH [27]. In a cell-free DNA end-joining assay, FEN1 was also found to be required for MH-mediated DSB repair in mammalian cells [44]. It was proposed that FEN1 endonuclease activity rather than its 5′ exonuclease activity could be involved in A-EJ; alternatively FEN1 may play other roles in A-EJ through its interaction with polymerases [89].

Addition of phosphate to the 5′-terminal end represents a limiting step for the repair of non-ligatable DNA ends. The major human 5′-DNA kinase activity relies on polynucleotide kinase (PNK) and hPNK directly interacts with the XRCC1 protein [90]. Given that the XRCC1/Lig3 complex is implicated in the PARP1-dependent A-EJ pathway [45], hPNK is suitable to play a role in A-EJ. In support of this idea, PNK is co-recruited to DNA ends with PARP1 and XRCC1/Lig3 and the ligation of 5′-OH terminal breaks is compromised in hPNK-depleted extracts [48].

3.2.3. DSB ligation

Lig4 associated with XRCC4 is dedicated to the repair of DSBs by the C-NHEJ pathway and there are no known function for this ligase outside this process [3]. Thus, much interest has focused on the possible role of the two other mammalian ligases (Lig1 and Lig3) for the joining of DNA ends in A-EJ.

Results from plasmid joining assays and biochemical experiments implicated Lig3 in A-EJ [33,45,47,91]. Structural evidence such as the presence of a zinc-finger domain which facilitates intermolecular ligation are compatible with its possible involvement in A-EJ [92]. Interestingly, ligation of incompatible ends by Lig3/XRCC1 is stimulated by MRN [33]. Lig3 has both nuclear and mitochondrial isoforms, the former associated with XRCC1 [93]. The recent development of mouse cell lines that are specifically deficient for the nuclear form of Lig3 has permitted investigation of its role in chromosomal translocation formation and A-EJ [94]. Nuclear Lig3-deficient cells show a reduced translocation frequency between two zinc-finger endonuclease-induced chromosomal DSBs, suggesting that Lig3-dependent A-EJ contributes to translocation events despite the presence of all C-NHEJ factors [94]. In contrast, Lig3 depletion through shRNA expression in wild-type or Lig4-deficient primary B cells or CH12F3 B cell lines does

not impair CSR assayed by IgA switching or formation of IgH/c-myc translocations [95]. However, these results may be explained by a low residual Lig3 level contributing to the A-EJ observed in Lig4-deficient cells and do not definitely rule out a dominant role of Lig3 in A-EJ [96].

XRCC1 is a scaffolding protein that stabilizes Lig3 so that Lig3 protein levels are drastically reduced in XRCC1-deficient cells [97]. Based on biochemical experiments, XRCC1 has been described as a mammalian A-EJ factor [33,45]. Another line of evidence implicating XRCC1 in A-EJ comes from its involvement in Ku-independent repair in *Arabidopsis thaliana* [98]. However, since plants are lacking a Lig3 homolog, *Arabidopsis* may function by recruiting other factors to carry out DSB repair in the absence of C-NHEJ.

Conditional XRCC1 inactivation in mature primary wild-type or XRCC4-deficient B cells does not impair A-EJ-mediated switch junctions (Sμ-Sγ1 and Sμ-Sε junctions), alter the frequency of IgH/c-myc translocations nor compromise the repair of I-SceI-induced chromosomal DSB [95]. Similarly, genetic ablation of XRCC1 in Lig4 deficient CH12F3 B cells does not reduce their ability to undergo CSR nor did it affect MH at switch junctions [99]. Together these results indicate that XRCC1 may not be required for A-EJ in the CSR context, although conflicting results have been reported [100]. In addition, Lig3 interaction with XRCC1 appears dispensable for A-EJ-mediated translocation in mouse cells [94] and XRCC1 mutation in a CHO cell line does not abrogate MH use measured using an extrachromosomal assay, also suggesting that XRCC1 is not required for A-EJ [32]. However, a contribution of XRCC1 to A-EJ of IR-induced breaks in G2 was recently reported in hamster cells [101]. Thus, the requirement for XRCC1 in A-EJ may vary in different models depending on whether DNA Lig3 activity is limiting or not in each corresponding situation.

Besides Lig3, Lig1 has also been implicated in A-EJ. Indeed, siRNA-mediated downregulation of Lig1 in human HTD114 cells leads to a reduction of MH-mediated end joining of linearized plasmids in cell-free extracts [91]. However, when DSBs are introduced at two loci by expressing zinc finger nucleases, Lig1 depletion in wild-type mouse cells do not have any effect on translocation frequency whereas Lig1 depletion in nuclear Lig3-deficient cells nearly abolishes translocations [94]. The involvement of Lig1 was further demonstrated in Lig3/Lig4 double mutant DT40 cells in which Lig1 contributes to the remaining alternative end-joining activity observed in these cells [102]. This implies that Lig1 may act as a back-up ligase for Lig3 in A-EJ.

These results may reflect the existence of at least two A-EJ pathways with Lig1 and Lig3 operating hierarchically, one Lig3-dependent pathway biased toward MH use, and a second Lig1-dependent pathway independent of MH [94].

4. Regulation of A-EJ

What makes A-EJ a marginal repair route for DSB under normal conditions?

The first likely reason is that the major C-NHEJ pathway relies on a champion DNA end binding factor, namely Ku. Ku is not only very abundant but also exhibits a very high affinity for DNA ends [3,103]. Nevertheless, PARP1 also binds to and is activated by DSBs *in vitro* [104,105]. Indeed, biochemical experiments provide evidence for a competition between purified Ku complex and PARP1 at DNA ends [61]. In cells however, the frequently associated SSBs after common clastogenic treatment (~20 fold ratio of SSBs to DSBs after IR) most probably divert PARP1 from DSBs [61]. PARP1 binding to, and activation by DSBs may further be down-regulated by DNA-PK activity [56,106]. However, it is conceivable that a marginal fraction of breaks that initially engaged in resection may escape C-NHEJ by preventing Ku loading and enter the A-EJ route [62,83,107] which may also support translocations in wild-type cells [81].

As compared to other C-NHEJ proteins, several findings support a major role of Ku for preventing access at DSBs to non C-NHEJ factors. For example in yeast, loss of either orthologs of Lig4 or XRCC4 has a milder effect on MRE11-dependent resection at DSBs than loss of Ku [108]. In plasmid-rejoining assays in human cells, Ku80 genetic ablation still allows a wild-type level of repair events. Inactivation of DNA-PKcs, Cer-XLF or Lig4, however, strongly inhibits repair, although this can be rescued by reducing the amount of Ku in these cells [109]. Similarly, joining efficiency of an intrachromosomal substrate is close to normal in Ku-deficient hamster cells but strongly reduced in XRCC4-defective cells [110]; in mouse embryonic fibroblats (MEFs), plasmid end-joining is decreased by XRCC4 defect but not by lack of Ku [111]. A PARP inhibitor compromised DSB repair in Ku-defective cells to a greater extent than in cells defective in other C-NHEJ factors [45,61,112]. In addition, we and others found that the recovery of A-EJ factors in damaged chromatin was stronger in the absence of Ku than in the absence of other C-NHEJ proteins [56,112].

A second reason for the marginal contribution of A-EJ to normal DSB repair is that C-NHEJ relies on a very efficient and flexible process, perfectly equipped to handle most of DSB classes [3]. This likely reflects the evolution of numerous protein/DNA and protein/protein interactions that optimize tethering, protecting, processing and end-joining activities necessary for repair of DSBs [3,13,113]. In addition, several results demonstrate functional cooperation within the C-NHEJ supra-molecular active complex so that each protein optimizes the activity of the other partners: for example, ligation proteins also optimize tethering [14] and processing of DNA ends [15,114]. Thus, A-EJ which rather appears as a composite process depending on the somehow fortuitous assembly of disparate components (see Fig. 1 and last section 6) cannot compete with the evolutionary selected C-NHEJ pathway. Accordingly, Iliakis's group has reported that while most IR-induced breaks are repaired with a fast kinetics by the C-NHEJ pathway (typical half life <30 min), the DSB repair that still occurred in C-NHEJ mutants that was not dependent on HR, proceeded much slowly ([115] and references therein).

The tight regulation of DSB resection acts as a key determinant in committing the repair of a DSB to either C-NHEJ or homology-based pathways including A-EJ (Fig. 2), which may also contribute to C-NHEJ predominance. Indeed, an admitted although optional trait of A-EJ is that it shares with HR a common initial resection mechanism promoted by the MRE11 nuclease and CtIP (see above, section 3.2.2.1). Furthermore 53BP1 is a major inhibitor of resection at DNA ends, operating through recruitment of at least RIF1 to its own ATM-dependent phosphosites [116–120]. The absence of 53BP1 increases end resection and channels repair to A-EJ in CSR [121]. Similarly, in U2OS cells transfected with linearized plasmids, RIF1 depletion leads to an increase in resection-based repair pathways including A-EJ [118]. Most probably through the same route, γH2AX or its binder MDC1 prevent CtIP-dependent resection and A-EJ at V(D)J DSBs in G1 murine lymphocytes [118]. Consequently, A-EJ shares with HR the cell-cycle regulation of resection, i.e. repression in G1 and expression in S/G2 phases through CDK activity (reviewed in [68] for HR). Indeed, the DSB repair defect in BRCA2-deficient cells is rescued by A-EJ under certain conditions [101]. A-EJ of IR-induced breaks or transfected plasmid in MEFs deficient for C-NHEJ and/or HR is efficient in G1, but further increased in G2 [122]. More recently, using a GFP-reporter A-EJ repair substrate, Truong and coll. showed that A-EJ requires cyclin-dependent activities and increases significantly as cells enter S-phase [83]. Together these studies suggest that A-EJ is active in all the phases of the cell cycle but in contrast to C-NHEJ, it increases as cells enter S-phase, concomitantly with CDK activity (Fig. 2). In addition, growth state also

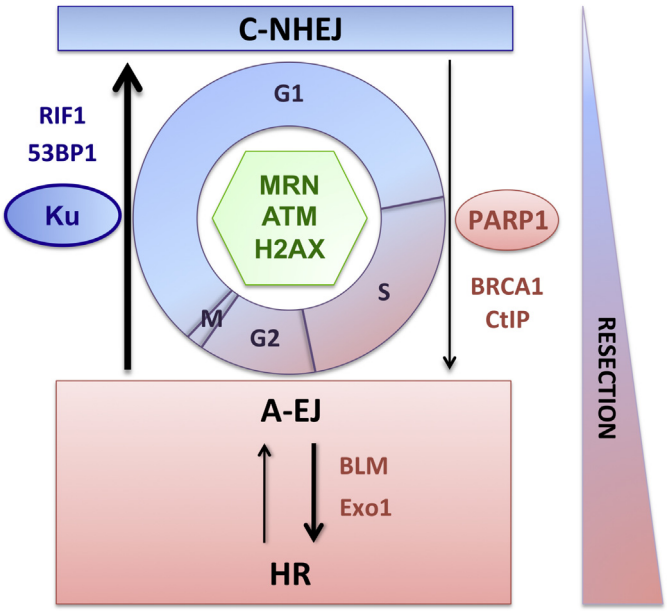


Fig. 2. Interplay between DSB repair pathways—Role of cell cycle and resection. C-NHEJ dominates both A-EJ and HR for the repair of DSBs, primarily by Ku competing (with PARP1) for the access to the breaks. Regulation of DNA end resection in a cell cycle-dependent manner is also critical for the repair pathway choice. In this respect, the ATM-dependent DDR pathway (MRN-ATM-H2AX) has no decision-making role *per se* but rather facilitates DSB repair by either C-NHEJ or HR, in G1 or S/G2 phases of the cell cycle, respectively. More specifically, activation of ATM leads to phosphorylation of 53BP1 and the subsequent formation of 53BP1/RIF1 complexes that accumulate at the breaks. In G1, 53BP1/RIF1 opposes BRCA1 and CtIP, thus limiting end resection and supporting C-NHEJ, particularly for complex DSBs and/or for DSBs arising in the heterochromatin. In S and G2, although C-NHEJ remains the predominent pathway, CDK1 phosphorylates CtIP enabling its association with BRCA1. The resulting BRCA1/CtIP complexes release RIF1 at least from a subset of DSBs, thus promoting end resection-dependent repair processes. The extension of resection carried out by EXO1 further bolsters up HR which dominates A-EJ. See the text for further details.

influences A-EJ since IR-induced DSB repair is markedly reduced in plateau-phase cultures of Lig4-null [123] or Ku-null [124] but not wild-type MEFs. In addition serum deprivation impacts on survival of C-NHEJ defective but not wild-type MEFs [125].

Finally, DSBs origin, complexity or context may influence the repair pathway choice. Concerning the origin of the DSB, mechanisms dedicated to avoid A-EJ operate at specialized breaks, like the ones occurring during V(D)J recombination. This process corresponds to a genetic rearrangement between exons encoding the amino-terminal variable regions of B- and T-cell receptors during lympocyte development [5,38,126]. It is initiated by RAG1/2 heterodimeric nuclease which introduces DSBs at cognate recombination signal sequences flanking the gene segments to recombine [5]. RAG-induced DSBs are specifically channeled to C-NHEJ as a result of several RAG properties. First, RAG expression is restrained to the G1 phase of the cell cycle [127], precluding resection-based V(D)J DSB repair mechanisms in normal conditions. Also, one side of each V(D)J DSB harbors a hairpin which requires processing mostly by Artemis, the C-NHEJ structure specific nuclease [128,129]. Finally, the RAG complex transmits the breaks specifically to the C-NHEJ machinery, so that in the absence of C-NHEJ core components, virtually no B or T cells can develop. This results in Severe Combined Immuno-Deficiency phenotype as described in mice deficient for XRCC4 [130–132], Lig4 [133], Ku80 [132,134–136], or Ku70 [137,138]. RAG-mediated bias for C-NHEJ is further substantiated by the observation that the repair of RAG-independent breaks generated by I-SceI-RAG fusion proteins is restricted to C-NHEJ [139]. Furthermore, RAG mutants have been described that enabled A-EJ to access and rejoin RAG-dependent DSBs, even in C-NHEJ proficient cells albeit to a lesser extent [140–142], resulting in increased translocation frequency and high incidence of lymphomas in a p53-null murine genetic background [143]. A-EJ restriction is dependent on a particular RAG2 subdomain [144]. Consequently, under normal conditions, the role of A-EJ in the V(D)J recombination is reduced to the smallest share since it is highly unlikely that the A-EJ machinery has a chance to encounter and deal with RAG-induced DSBs.

Increasing complexity of DNA ends also may affect the rate of the reaction, the requirement for processing events including resection, and thereby influence the relative contribution of C-NHEJ, HR and A-EJ. Early *in vitro* experiments documented the influence of DNA end compatibility on both the efficiency and accuracy of C-NHEJ and A-EJ [30,31], as well as the position of MH relative to the ends on the propensity to perform MMEJ [31,44,145]. The presence of repetitive sequences in the vicinity of the break may favor A-EJ over C-NHEJ and HR. This notion is consistent with the necessity of tightly silencing A-EJ at the telomere, and with the ability of A-EJ to significantly rescue C-NHEJ deficiency during the CSR process in which DSBs are formed in regions containing repeated motifs (see the following sections 5-1 and 5-2).

In summary, although A-EJ is largely dominated by both the C-NHEJ and HR pathways, it can serve as a backup pathway not only for C-NHEJ, particularly in G1 when HR is inoperative, but also in G2 for HR when extended resection precludes C-NHEJ activity.

5. Physiological relevance of A-EJ

5.1. Class-switch recombination (CSR)

CSR is a cellular process occurring in peripheral IgM-expressing B lymphocytes (mature B cells) in response to antigen-dependent stimulation. It is an intrachromosomal deletional recombination requiring two initial DSBs (like V(D)J recombination). CSR is responsible for exchanging the immunoglobulin isotype from IgM to IgG, IgA or IgE by recombining the corresponding Ig constant chain exons, thus generating antibodies with different effector functions [6,38,146]. In contrast to DSBs that arise during the V(D)J recombination, CSR-dependent DSBs are not produced by a site-specific recombinase but are generated by a complex mechanism initiated by the Activation-Induced Cytidine Deaminase (AID) [6,146]. AID operates at both the donor Sμ switch region (upstream the Cμ constant region) and one acceptor S region adjoining the heavy chain constant region that is to undergo recombination. Sμ, Sα and Sε regions are composed of GC-rich pentamer tandem repeats, whereas Sγ regions mainly contain larger tandem repeats [147]. Transcription at S regions facilitates AID-dependent deamination of deoxycytidines in single-stranded DNA, resulting in deoxyuridines that are subsequently processed by the BER pathway *via* the Uracil N-glycosylase (UNG), giving rise to SSBs [6]. Proximal SSBs on opposite strands, like those arising at pentamers containing a GC motif, then result in DSBs [148].

DSBs generation during CSR is not believed to be particularly constrained to C-NHEJ as compared to the V(D)J recombination (see above, section 4). Indeed, when V(D)J abrogation in C-NHEJ deficient mice is artificially circumvented to allow production of mature B cells, it appears that, although affected, substantial CSR can occur in murine lymphocytes deficient for XRCC4 or Lig4 [149–152], DNA-PKcs [153–157], Artemis [156,158], Cer-XLF [159], and also Ku70 or Ku80 [149]. Consistent with a role for A-EJ in operating during CSR in the absence of C-NHEJ, the frequency and length of MH at S-S junctions are enhanced [149,151,152,159]. Similar results are found in Sμ-Sα junctions in cells from patients deficient for Lig4 [160], Artemis [158,161] and Cer-XLF [162]. However, Sμ-Sγ junctions are almost normal, suggesting that the ability of A-EJ

to use MH depends on the degree of homology between S regions. Of note, Lig4 mutations in patients are hypomorphic [160] and, in the case of Artemis, Sμ-Sγ junctions could not be recovered from "Artemis-null" patients and were only found in a patient carrying a hypomorphic mutation [161]. Hence the extent of homology at the S-S junction may influence the capacity of A-EJ to compete with residual C-NHEJ for the rejoining of S regions, allowing only Lig4 or Artemis hypomorphic mutants to reseal some poorly homolog Sμ-Sγ pairs.

Besides the less frequent S-S junctions in C-NHEJ deficient B cells, non-productive recombination events may arise during CSR following the rejoining of two AID-dependent DSBs within a given S region, which results in internal switch deletion (ISD). Compared to ISDs occurring in wild-type B cells, ISDs in C-NHEJ deficient B cells are recovered at a much higher frequency and the junctions exhibit a more pronounced MH usage [163]. These observations indicate that A-EJ is more prone to catalyze short-range intrachromosomal reactions than standard long-range reactions (as well as interchromosomal translocations, see below, section 5-3). This phenotype likely accounts for the increased opportunity for A-EJ to find and use micro-homologous sequences within a given S region as compared to between different S regions.

Apart from the dominant activity of C-NHEJ over A-EJ, the tethering of distal S regions necessary for functional CSR may also influence the DSB repair pathway choice. Indeed, in contrast to the moderate role of the ATM-dependent DSB response pathway in V(D)J recombination, efficient CSR was found to require MRN [71,74,164–166], ATM [165,167–169], H2AX [170–174], MDC1 [171,175], RNF8 [176–178], RNF168 [177,179], 53BP1 [121,180–183] and RIF1 [116–120]. This probably reflects the necessity to stabilize the synapsis between two distant DSBs in the absence of a RAG-like complex. Inactivation of any of these DSB response factors results in CSR impairment and genomic instability, albeit to varying degrees. 53BP1 deletion apparently leads to the most severe defect, by preferentially affecting long-range S-S recombination whereas non productive ISDs are enhanced [180,182]. In addition to stabilizing the synapsis, DNA damage response factors also promote efficient CSR by protecting DNA ends from degradation in a distance-independent manner as shown for 53BP1, thus further favoring C-NHEJ [121]. In agreement, in 53BP1, ATM or H2AX deficient mouse B cells, S-S junctions exhibit increased deletion and MH usage, indicative of end processing by A-EJ [121,167,180]. However, in the case of poorly homologous Sμ-Sγ1 junctions, no MH change [169] and increased MH have been reported [167] in the absence of ATM, although identical mouse strains were used in both experiments. Among hypotheses to explain this discrepancy are the cell source and, more likely, the stimulation protocol. In this regard, CD40L + IL4 [167] would be more suitable than LPS + IL4 [169] for studying MH variations at Sμ-Sγ1 junctions. Accordingly, when MH usage was analyzed upon XRCC4 conditional deletion in mouse B cells, no change was observed when switching to IgG1 with LPS + IL4 [151], whereas the average junctional MH was increased by three fold when using CD40L + IL4 [152]. This indicates that both the S-S combinations and the stimulation protocol chosen are to be considered when interpreting MH usage and the supposedly related A-EJ activity in CSR. CSR junctions were also analyzed in human patients deficient for various components of the DNA damage response. In Ataxia telangiectasia (AT) patients, MHs were notably increased at Sμ-Sα junctions, even over 10 bp and, less markedly, at Sμ-Sγ junctions [165,184]. In NBS1 (Nijmegen breakage syndrome, NBS) and MRE11 (AT like disorder, ATLD) deficient patients, milder CSR defects are reported with still increased MH usage and significantly more Sμ-Sα junctions flanked by long imperfect repeats [164,165]. Worthy of note is the pattern of MH usage found in ATR deficient patients (Seckel syndrome) featuring increased 4–6 bp MH but normal MH usage for longer stretches, in contrast to ATM [184], probably accounting for specific role of ATM and ATR in regulating limited and extended resection, respectively.

Paradoxically, ATM inhibition reduces the CSR defect in 53BP1 deficient mouse cells by decreasing resection [121]. This suggests that, as in V(D)J recombination, ATM assumes antagonistic roles in stabilizing the synapsis and favoring end resection, thus promoting C-NHEJ and A-EJ, respectively. In this respect, the MRN complex also appears to be a multifunctional factor, involved both in synapsis stabilization through its bridging function, and in the regulation of end resection [66]. In mouse B cells, MRE11 deletion profoundly impaired CSR (more than ATM deletion) and led to slightly less MH at Sμ-Sε junctions. In contrast MRE11 nuclease activity was partly dispensable for CSR and its inactivation resulted in unchanged MH usage at S-S junctions, [71]. In this context and constrasting with MRN, CtIP seems to only promote resection during CSR. Its knockdown results in less MH usage both in Ku70-deficient and in wild-type murine B cells [82]. This indicates that C-NHEJ can accommodate to some extent to CtIP-dependent resected DNA ends and/or that A-EJ may contribute to normal CSR. Recently, CtIP or EXO1 inactivation was shown to only partially abrogate resection and rescue CSR defect in 53BP1-null mouse B lymphocytes, supporting their role in A-EJ, but also revealing their functional redundancy or the existence of additional resection mechanism operating during A-EJ [185].

As for a role of A-EJ in normal CSR (i.e. in the presence of C-NHEJ), some S junctions in C-NHEJ proficient human [160] and murine B cells frequently use MHs above four nucleotides [149,152], consistent with A-EJ repair products. Furthermore, ISDs in C-NHEJ proficient cells [160] also exhibit frequent use of MH and were proposed as a readout of A-EJ activity [149,152]. However, repetitive sequences in S regions constitute a breeding ground for any MH-mediated end-joining process, making delusive any interpretation about the respective contribution of A-EJ or C-NHEJ on the sole basis of MH usage. Investigating the role of nuclear Lig3 in both S-S junctions and ISDs should therefore prove highly informative in evaluating the actual role of A-EJ in CSR.

5.2. A-EJ and telomeres

A major challenge for eukaryotic cells is to prevent the ends of their linear chromosomes from recognition as DSBs by the DNA repair and signaling machinery. In most eukaryotes, specialized nucleoprotein complexes, called telomeres, protect chromosome ends.

In mammals, telomeric DNA consists of a double-stranded region, based on the repetition of the hexameric TTAGGG sequence, extended by a 3′ protruding single-stranded G-rich sequence, the G-tail or telomeric overhang. Telomeric DNA is bound by a specific telomeric complex called shelterin that is composed of TRF1, TRF2, RAP1, TIN2, TPP1 and POT1 proteins. Whereas TRF1 and TRF2/RAP1 proteins are associated with double-stranded telomeric DNA, TPP1 and POT1 proteins associate with the G-tail. TIN2 protein bridges together both complexes (reviewed in [186]).

The protection of chromosome ends by telomeres is a multilayer response in which distinct telomeric factors repress the activation of different signaling and repair pathways. From biochemical data based on the rejoining of a linear plasmid mediated by cellular extracts or purified proteins, we have proposed a double lock model against end joining at telomeric ends in which the TRF2/RAP1 complex inhibits C-NHEJ, whereas the DNA–PK complex blocks A-EJ [187]. This model was further substantiated and refined by data obtained in cells [63,84,188]. Several reports have characterized the first lock level, demonstrating that different shelterin components sustain different protective roles. TRF2 directly binds ATM and blocks its activation [189–192]. Additionally, TRF2 anchors

RAP1 to telomeres, thereby preventing telomeric fusions by C-NHEJ [193,194]. Biochemical studies in our lab have demonstrated that TRF2/RAP1 prevents proper Ku binding and DNA-PK activation at telomeric ends [187]. Thus, removal of TRF2 from telomeres provokes end-to-end chromosome fusions [195,196], which are mainly dependent on Lig4 activity [188,195,197,198]. Moreover, these C-NHEJ dependent end-fusions are completely abrogated in an ATM deficient background [190] and require the C-NHEJ facilitator 53BP1 [84]. ATM activation ensures 53BP1 recruitment and phosphorylation together with its association with RIF1 and PTIP, both to promote long range end-joining [199] and to prevent resection at DNA ends [116,120,154,200].

In contrast to TRF2, it has been shown that TPP1-POT1 prevents ATR activation at telomeres since removal of TPP1-POT1 complex activates ATR signaling, independently of the status of the TRF2 protein [84,190–192]. In a murine 53BP1-null background, overexpression of a dominant negative form of TPP1, which globally destabilizes the shelterin [192,201], induces telomeric fusions that are completely abrogated in ATR depleted cells and independent of Lig4, thus relying on A-EJ; moreover, further depletion of TRF2 in this model considerably increases the number of telomeric fusions [84]. Accordingly, total depletion of the shelterin complex in Lig4$^{-/-}$ MEFs yielded significant telomere fusions by A-EJ [63]. Together these findings show that TRF2 and TPP1/POT1 proteins cooperate to fully inhibit A-EJ at telomeric sequences.

Concerning the second lock at telomeres, several reports support a role for DNA-PK as a protection factor against A-EJ. Spontaneous chromosome-end fusions are promoted by a deficiency in either Ku [188,202–207] or DNA–PKcs [208,209], while at least in the absence of Ku, the core shelterin complex appears broadly normal [197]. Furthermore, depletion of Ku in TPP1/POT1 depleted cells or in shelterin-free telomeres provokes a dramatic increase of A-EJ dependent chromosome-end fusions [63,84].

What are the factors involved in A-EJ acting on dysfunctional telomeres? Consistent with the admitted players in A-EJ, inhibition and/or inactivation of PARP1 or Lig3 proteins significantly reduced the fusions of shelterin-free telomeres [63]. In addition to the ATR activation [84,190] which may act at an early step, a nucleolytic process is required. Indeed, end-to-end fusions induced by TPP1/POT1 depletion are reduced to a background level in the absence of the CtIP protein [84]. 53BP1 functions as a brake on resection since 53BP1 depletion in cells with shelterin-free telomeres provokes an important lengthening of the 3′ overhang, which is dependent on BLM, EXO1 and CtIP proteins [63]. Taken together these results suggest that shelterin components are required to block nucleolytic degradation of telomeres and in the absence of shelterin, 53BP1 further inhibits this degradation. A major role for Ku in protection of telomeres from exonucleolytic attack in yeast and plants has been described [210–214]. Notably, Ku complex appears to elicit only a moderate protection against end resection at deprotected telomeres compared to the major effect of 53BP1 [63]. However, prevention of A-EJ by 53BP1 concerns telomeres already engaged in damage signaling contrarily to Ku constitutively present at all telomeres and which does not rely on damage signaling to counteract A-EJ.

Aside from A-EJ function at chromosome ends following artificial induction of telomeric dysfunction, what is its impact on naturally eroded telomeres? The maintenance of the telomeric sequence is ensured by the telomerase, a specialized ribonucleoprotein complex that counteracts the natural telomere attrition occurring at each cell division. In the absence of telomerase, mammalian telomeres shorten from 50 to 100 bp per cell division [215]. When reaching a critical size, telomeres no longer protect chromosome ends from the DNA Damage Response and are recognized as DNA breaks. Eroded telomeres participate in end-to-end chromosome fusions and induce cell cycle arrest, senescence or apoptosis [216].

In 2002, Espejel and co-workers have shown that chromosome end fusions in late generation telomerase-null mice, bearing critically shortened telomeres, are dependent on Ku80 and DNA-PKcs proteins [217]. However, experiments from DePinho's lab show that end-to-end chromosome fusions with critically shortened telomeres occur in the absence of Lig4 and DNA PKcs proteins and could involve A-EJ [218], in line with the report of Ku- and Lig4-independent telomere fusions on telomere attrition in fission yeast and in plants [214,219,220]. Molecular characterization of junction sequences of critically shortened human fused telomeres, present regions of microhomologies as well as extensive telomere deletions, both features reminiscent of A-EJ [221]. Finally, Rai and co-workers demonstrated that naturally eroded telomeres were prone to fuse despite the absence of the critical 53BP1 factor for C-NHEJ, indicating that A-EJ operated [84]. Taken together these data argue for a contribution of A-EJ to the processing of naturally eroded telomeres.

5.3. Contribution of A-EJ to chromosomal rearrangements and cancer

Genomic instability is a prominent hallmark of cancer that can determine the acquisition of multiple additional cancer capabilities [222]. Among various forms of genomic instability, chromosomal instability has been incriminated as a causative factor in cancer development for a century [223]. This view is fully supported by the discovery of the Philadelphia chromosome, that is the recurrent t(9;22) translocation associated with chronic myeloid leukemia [224]. Although mutations affecting the sequence of genes are more frequent than chromosomal rearrangements, translocation are considered to drive 20–40% of cancers [225,226]. Translocations are a subtype of chromosomal aberrations in which parts of two different non-homologous chromosomes are fused. Recurrent translocations are commonly associated with haematopoietic malignancies [173,227,228], although increasing amount of data confirms their implication in solid tumors as well [225]. In most cancer cells, chromosomal aberrations are multiple and complex, making it sometimes difficult to ascertain whether a particular rearrangement is a passenger mutation or an oncogenic driver. The fine analysis of chromosomal rearrangements has greatly benefited from recently developed powerful tools allowing genome-wide translocation mapping and large-scale analysis of spatial nuclear organization [229].

The respective contribution of C-NHEJ and A-EJ in the origin of translocations has been the subject of intense research in different contexts and this issue is still being debated.

5.3.1. End-joining processes in chromosomal rearrangements

C-NHEJ may account for translocation events in some specific situations such as during the recovery from early apoptotic DNA fragmentation [230–232], or in the pathogenesis of acute leukemias secondary to cancer treatments based on Topoisomerase-II (TopoII) inhibitors that frequently feature translocations involving the mixed-lineage leukemia (MLL) gene [233,234]. The role of C-NHEJ in chromosomal rearrangements has been more clearly established in the special case of one-ended DSB repair following replication fork collapse [235–238], but a recent study also suggests a significant role for A-EJ in the repair of one-ended DSBs following hydroxyurea replication fork arrest [83].

Apart from these particular situations, most studies suggest both a suppressive role for C-NHEJ core components and a prominent role for A-EJ in mediating translocations. Indeed, spontaneous chromosomal rearrangements including translocations are more frequent in Ku80-, Ku70-, Lig4- or Cer-XLF-deficient murine

fibroblasts or ES cells [239–242]. This effect is exacerbated upon DSB induction by ionizing irradiation [240]. The use of reporter systems in mouse ES cells confirm and extend this observation. Depletion of Ku70 [81,243,244], XRCC4 [94,243] or Lig4 [94,243], or DNA-PKcs inhibition [245] strongly increases translocation frequency without significantly affecting the pattern of MH length at the breakpoint junctions when tested [81,94,243,246]. In C-NHEJ deficient mice, cancer susceptibility is only slightly enhanced, unless genomic gatekeepers like p53 are also deleted. Interestingly, nearly all C-NHEJ/p53 double deficient mice die from pro-B lymphomas mostly associated with recurrent translocations involving the immunoglobulin heavy chain locus (IgH) and c-myc [227]. High incidence of pro-B lymphomas has been described in mice deficient for both p53 and Ku80 [239,247], Ku70 [248], DNA-PKcs [249–251], Lig4 [252], XRCC4 [253], and Artemis, although with different chromosomal rearrangements in the latter case [254]. Cer-XLF/p53 double deficient mice do not exhibit high incidence of pro-B lymphomas [159], likely reflecting functional redundancy with other factors such as ATM, H2AX and 53BP1 during V(D)J recombination [255–257].

Recurrent translocations associated with pro-B lymphomas in C-NHEJ/p53 double deficient mice likely result from aberrant repair of RAG-induced DSBs. The additional deletion of RAG suppresses the pro-B lymphoma high incidence in DNA-PKcs/p53 [258,259]), Ku80/p53 [248] or XRCC4/p53 double-deficient mice [260]. When analyzed, translocation breakpoint junctions revealed MH usage with no direct joints [248,260], further supporting the propensity of A-EJ for catalyzing illegitimate end-joining events.

In the absence of functional C-NHEJ, the A-EJ pathway is also implicated in chromosomal rearrangements following AID-induced DSBs during CSR. Although quite robust in replacing C-NHEJ during CSR (see above, section 5-1), A-EJ does not appear fully adequate since B cells show frequent DSBs and translocations [152,156]. The conditional deletion of XRCC4 in mature B-cells of p53-null mice leads to peripheral B-cell lymphomas that mostly rely on translocations between IgH and c-myc associated with MH at the junction [261]. This observation further confirms the critical role of C-NHEJ in suppressing A-EJ mediated translocations during CSR. A role for AID-induced DSBs in IgH/c-myc translocations was subsequently established [262]. Complex translocations in pro-B lymphomas from RAG-induced DSBs in p53/C-NHEJ double deficient mice routinely harbor c-myc and IgH amplifications [248,260]. In contrast, translocations found in peripheral B-cell lymphomas originate from reciprocal rearrangements without c-myc amplification, reminiscent of translocation events in Burkitt's lymphoma which also feature MH at the junction [263]. Furthermore, the evidence of AID-induced DSBs both in IgH and c-myc [264] establishes the important role of AID in promoting IgH/c-myc translocations even in C-NHEJ proficient contexts [173,264].

Recently developed techniques for genome-wide translocation sequencing or analysis of chromatin spatial conformation indicate that both the frequency of DSBs at off-target locations by AID (or RAG in immature B-cells) and the spatial proximity between broken chromosomal regions contribute to the high frequency of IgH/c-myc translocations in B-cells during the CSR (or the V(D)J recombination) [265–268]. What are the respective roles of A-EJ and C-NHEJ in catalyzing IgH/c-myc translocations? Although the contribution of A-EJ is clear in the absence of functional C-NHEJ, the choice between the two pathways remains unclear in wild-type B-cells. Nearly all translocation breakpoints use 1–7 bp of MH when DSBs are induced by I-SceI both in c-myc and IgH, similar to what is found in C-NHEJ-deficient B-cells [264]. In contrast, when DSBs are produced by I-SceI and AID in c-myc and IgH, respectively, about half of the junctions are direct and the overall MH usage is under 1 bp, reminiscent of the junctions observed during normal CSR between switch regions [152]. This result is consistent with a major role for C-NHEJ in the formation of translocations during CSR. This may also reflect a form of coupling between AID-mediated cleavage and the C-NHEJ machinery, although less stringent than between RAG-induced DSBs and C-NHEJ. This notion is further supported by the different ability of Sμ compared to other S regions to participate in translocations [269]. Alternatively, the mechanism of production of DSBs, by affecting the nature of DNA ends, may influence the way they are processed by the A-EJ machinery, thus giving rise to more or less MH at the junction.

Outside the context of CSR, by using translocation reporter systems in mouse, the unmodified pattern of MH usage whatever Ku or Lig4 status, strongly argues in favor of a prominent role for A-EJ in catalyzing translocations [81,94,243]. Importantly, this method also provides compelling evidence relative to the mechanism of A-EJ. It has established the role of CtIP in the early resection step [81], as well as the major role of Lig3 in translocation formation, including its nearly full dominance over Lig1 and an apparent dispensability for XRCC1 [94], consistent with other studies [32,95]. In addition the role of PARP1 in promoting translocations was also evidenced, both in murine and human cells, following nuclease-, IR- or etoposide-induced DSBs [245], or in the context of CSR [62].

5.3.2. End-joining processes in cancer

So far in human tumors, the estimated contribution of A-EJ to translocation formation mainly rely on indirect evidence based on MH at breakpoint junctions commonly observed in haematological malignancies [227,228] and also in various solid cancers [270–272]. In support of a role of A-EJ in carcinogenesis, several studies suggested the notion that the balance between C-NHEJ and A-EJ might be shifted toward the latter in tumor cells, thus accounting for genetic instability. Recently, using an integrated A-EJ reporter system, tumor cells where shown to use significantly more A-EJ than non tumor immortalized cells [83], consistent with several earlier observations. For instance, BCR-ABL-positive chronic myeloid leukemia (CML) cells overproduce reactive oxygen species (ROS) and repair ROS-induced DSBs through error-prone pathways including end-joining with large deletions [273]. This observation was extended and supported by showing reduced protein expression level of Lig4 and Artemis in CML cell lines together with an up-regulation of Lig3 and WRN proteins, both being involved in a substantially active end-joining subpathway which uses MH [274]. Similarly, FMS-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD)-positive acute myeloid leukemia cells also accumulate more ROS and subsequent DSBs [274]. Moreover, compared to control cells expressing wild-type FLT3, FLT3/ITD-positive cells resort partly to a low-fidelity MH-based end-joining process for repairing DSBs, in line with both an increased Lig3 and a decreased Ku expression levels [275]. Both deletion extent and MH usage were reduced upon treatment with an FLT3 kinase inhibitor or by down-regulating Lig3 expression level, suggesting an increased contribution of A-EJ in DSB repair in FLT3/ITD cells [275]. Cells from FLT3/ITD knock-in mice essentially exhibited similar differences in end-joining characteristics and protein expression pattern, compared to wild-type cells [275]. More recently, in murine FLT3/ITD-positive pro-B cells, a PARP1 overexpression was also described concomitantly with a decrease of Ku expression which resulted in a DSB repair defect and impaired V(D)J recombination that could be alleviated upon inhibition of FLT3 [276].

Together, these results indicate that, through an hitherto unclear mechanism, oncogenic tyrosine kinases may be critical in certain circumstances for regulating the balance between C-NHEJ and A-EJ in cancer cells, thus promoting genomic instability. In line with this notion, tumor progression and agressiveness were also found correlated with low DNA-end binding activity of Ku in basal

cell carcinomas [277], as well as in breast and bladder cancers [278]. Interestingly, in both studies, early stages of cancer rather appear to correlate with an increased Ku activity, thus suggesting a two-phase dynamics in cancer progression with an early C-NHEJ overactivity and a late shift toward A-EJ. Independently, DSB repair activity was further analyzed in several bladder cancers with an *in vitro* end-joining assay that demonstrated in high-grade tumors a preference for using a C-NHEJ-independent, error-prone and MH-mediated DSB repair pathway [279]. Strikingly, a more recent proteomic analysis on several B-cell samples from patients with Waldenström's macroglobulineamia revealed a strong down-regulation of Ku70 which pathophysiological meaning remains to be evaluated [280].

Altogether these data provide a rationale for designing novel anticancer chemotherapy targeting A-EJ. Recent papers described a promising strategy combining both existing PARP1 inhibitors and newly developped Lig3 inhibitors to preferentially kill hormone-resistant breast cancer cells [281] or CML cells resistant to BCR-ABL-directed tyrosine kinases [282] that exhibit elevated PARP1 and Lig3 expression levels. C-NHEJ and A-EJ components might then be considered as novel biomarkers to identify candidate tumors potentially responding to therapy targeting either C-NHEJ (mainly through inhibition of DNA-PKcs [12] or Lig4 [283]) or A-EJ. Alternatively, adjuvant therapy based on PARP1 inhibitors may help contain chromosomal rearrangements mediated by A-EJ during conventional cancer treatment.

Overall, mounting evidence support a key role for A-EJ in chromosomal instability including translocation formation, which may represent the most relevant biological impact of this pathway. However, although MH is frequently found at translocation breakpoint junctions in haematological malignancies and in solid cancers, evaluating confidently the contribution of A-EJ in carcinogenesis will require a sharper landscape of the mechanism and specific components of this pathway.

6. Concluding remarks

6.1. A-EJ: a single pathway or multiple pathways?

During the past decade, considerable effort has been made to unravel the physiological functions of A-EJ and to elucidate its underlying molecular mechanism. Nonetheless, despite the obvious dependence on Lig3 and/or Lig1 and a trend toward MH usage relying on resection promoted by MRN and CtIP, no specific genetic or mechanistic attributes have been conclusively established so far (see Fig. 1). Moreover, regarding the reliance on MH, two different A-EJ pathways have been proposed based on experiments on CSR in the absence of XRCC4/Lig4, Ku70 or both [149,163]. Loss of Lig4 (or XRCC4) results in CSR junctions harboring almost exclusively MH, while 10–15% of junctions retain direct joints in Ku70 or Ku70/Lig4 deficient B cells. From these findings A-EJ was proposed to encompass at least two pathways: (i) a Ku-dependent/Lig4-independent pathway relying solely on MHs, (ii) a Ku-independent/Lig4-independent pathway still capable of carrying out some direct end joining [149,163]. However, it is also conceivable that the different CSR outcomes result from the necessity for a single A-EJ complex to overcome the binding of Ku at DNA ends when present, potentially through an MRN-dependent internal nucleolytic attack as reported in yeast [284]. Therefore, somewhat counterintuitively, under C-NHEJ deficiency conditions but in the presence of Ku (absence of XRCC4/Lig4), joining would require removal of the abortive C-NHEJ complex by resection, leading to an increased use of MHs, while in the absence of Ku, DNA ends would be more accessible to direct joining.

From the aforementioned studies and on the basis of genetic analysis of translocation mechanism in mouse cells [94] together with *in vitro* end joining assays investigating the relative use of MHs by Lig3 and Lig1 [91], it was further speculated that Lig3 and Lig1 might specifically operate in the Ku-dependent/Lig4-independent and the Ku-independent/Lig4-independent A-EJ pathways, respectively. However, both in mouse and chicken cells and with different sources of DSBs, Lig3 appears to largely dominate Lig1 which serves at best as a backup ligase when both Lig4 and Lig3 are absent [94,102].

Consequently, since many other interpretations are also possible, further experiments are needed to unequivocally establish whether A-EJ rely on a single pathway or on various (sub)pathways depending on the context. Alternatively, a simpler possibility still exists that A-EJ would not be a genuine repair pathway at all.

6.2. A-EJ: the "no pathway" option

Several lines of evidence argue against A-EJ being a true repair pathway.

First, as said before, the putative A-EJ mechanism is still ill-defined, although resembling a combination of components from the HR and/or SSA pathways as well as from the excision repair machineries such as BER or NER (Fig. 1). Moreover, A-EJ seems highly versatile with respect to the subsets of proteins potentially involved, the nature of the breaks used as substrates and the variety of sequence, chromatin and cellular contexts in which alternative end-joining can operate. The A-EJ flexibility is also reflected by the repair outcome (itself influenced by the previously mentioned elements) ranging from direct and accurate rejoining to deletional repair events, with or without MHs or insertions at the junction, and ultimately to illegitimate rejoining reactions resulting in translocations and other chromosomal rearrangements. Although not strictly dependent on MH, the bias toward MH may provide some stability between DNA ends, thus potentially compensating a lack of protein factors specifically devoted to carrying out a synapsis during A-EJ. In fact the absence of stable synapsis appears to be the weak point of A-EJ and may explain the low fidelity of this process.

Second, no relevant physiological function has been uncovered for A-EJ which seems not to have carved out a place to operate as a first choice pathway. Much to the contrary, A-EJ is highly error-prone both at the sequence level and in legitimately rejoining DNA ends, leading to frequent DNA loss at the junctions and to chromosomal rearrangements, respectively. As a result, the only appreciable cellular impact of A-EJ rather relies on pathological events, as discussed in this review. CSR is no exception, and even if AID-induced DSBs in S regions would appear fairly amenable to repair by A-EJ, C-NHEJ is necessary for efficient CSR and cannot be adequately replaced by A-EJ. Recently, variations between relative C-NHEJ and A-EJ activities assessed *in vitro* with cell free extracts prepared from mouse embryos at different stages of development, suggested a role for A-EJ during embryogenesis [285]. However *in vivo* experiments are required to confirm and confidently interpret these findings.

Finally, in non pathological situations and in wild-type genetic contexts, A-EJ activity is limited; C-NHEJ largely dominates other pathways at two-ended DSBs, while HR specifically deals with one-ended DSBs arising in S phase and with some two-ended complex DSBs lying in heterochromatin in S/G2.

Consequently, cells seem to have evolved to stave off as much as possible any attempt to deal inadequately with free DNA ends, particularly by setting up safe synapses soon after DSB appearance, or even before, during physiological processes such as V(D)J recombination, CSR and meiosis (Fig. 3). The core components of C-NHEJ provide the basis for this synapsis which is further strengthened by partially overlapping or complementary factors like chromatin modifications, MRN or 53BP1 bridging activities, as well as more

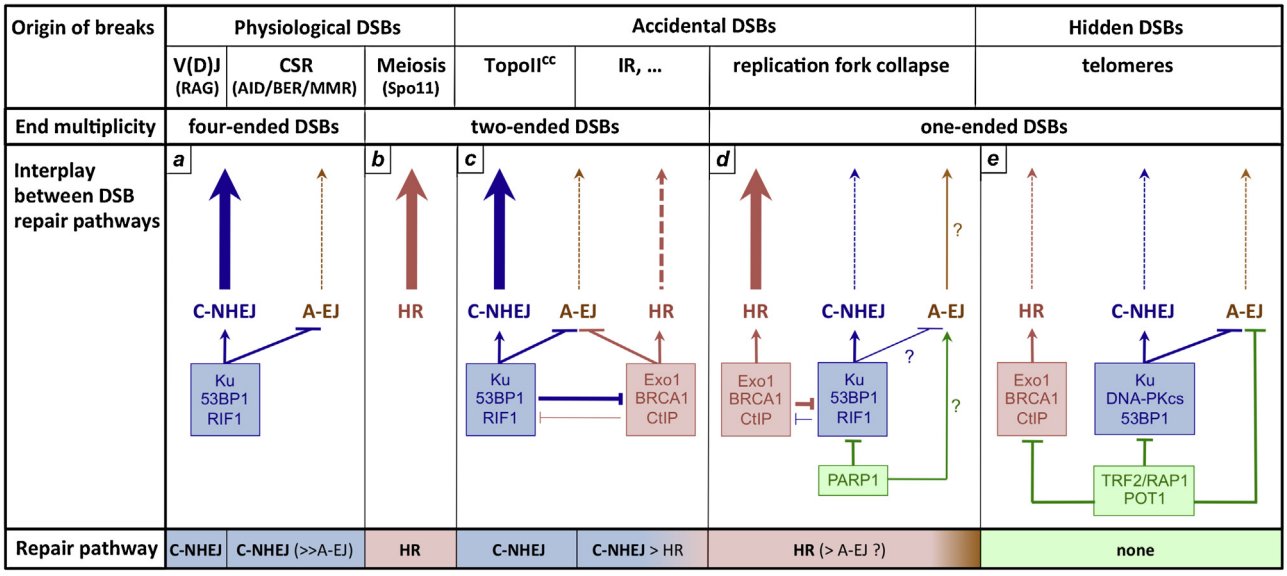


Fig. 3. Interplay between DSB repair pathways—Influence of the origin and nature of breaks. (a) Physiological four-ended DSBs are formed in G1 and are tightly shepherded to C-NHEJ, particularly RAG-dependent breaks arising during V(D)J and to a lesser extent AID-dependent breaks during CSR. C-NHEJ fully dominates A-EJ although A-EJ can partly rescue CSR when C-NHEJ is ablated. Note that in case of C-NHEJ deficiency, persistent CSR-mediated DSBs can also be repaired by HR in G2. (b) Meiotic DSBs are channeled to HR which is further supported by the lack of Ku and 53BP1 protein expression at the time point of meiotic recombination. (c) Accidental two-ended DSBs are essentially repaired by C-NHEJ, especially in the case of TopoII cleavable complexes (TopoII^{cc}). In S and G2, HR may also contribute to the repair of a subset of DSBs. In any case, A-EJ appears largely dominated by C-NHEJ and HR (see also Fig. 1). (d) Repair of one-ended DSBs in S phase (replication fork collapses), when BRCA1/CtIP complexes dominate 53BP1/RIF1, is performed by HR-mediated pathways. PARP1 also participates in C-NHEJ suppression thus promoting HR and potentially A-EJ which may contribute to some repair events. (e) Telomeres are protected from unwanted fusions through a multiple lock mechanism primarily performed by the shelterin complex which suppresses HR, C-NHEJ and A-EJ, while DNA-PK also silences A-EJ. For each situation, the resulting predominent repair pathway is indicated. Color code: blue = C-NHEJ, red = HR, brown = A-EJ, green = no repair.

specifically RAG1/2 during the V(D)J recombination and TopoII itself when failing or when blocked as a cleavable complex. Telomeric ends are also protected from end joining by A-EJ through a double lock involving the shelterin complex and DNA-PK.

As a result, A-EJ may be viewed as an opportunistic catch-all process using any available activity to reseal escaped DNA ends when possible; this bricolage operates at the expense of genetic stability. However, in line with its early suggested backup function [28], A-EJ could be of some utility as a last-ditch attempt to join DNA ends when C-NHEJ and HR pathways are overwhelmed with too many DSBs. Conceivably, another long-term beneficial aspect of A-EJ would concern genome plasticity and evolution. In this respect deletions represent the most frequent mutational events and might potentially be contributed by A-EJ [286]. Interestingly, intron loss during evolution in metazoans was recently reported to rely on MH-mediated end joining [287].

Whether or not it is a genuine DSB repair pathway, A-EJ is emerging as an important cellular process in several pathological situations and its full characterization remains a major research area for the coming years.

Conflict of interest

The authors declare no conflict of interest.

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