Role of alternative end-joining in genome stability

Repair of DNA double-strand breaks by mammalian alternative end-joining pathways

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ABSTRACT

Alternative end-joining (a-EJ) pathways, which repair DNA double-strand breaks (DSBs), are initiated by end resection that generates 3′ single strands. This reaction is shared, at least in part, with homologous recombination but distinguishes a-EJ from the major nonhomologous end-joining pathway. Although the a-EJ pathways make only a minor and poorly understood contribution to DSB repair in nonmalignant cells, there is growing interest in these pathways, as they generate genomic rearrangements that are hallmarks of cancer cells. Here, we review and discuss the current understanding of the mechanisms and regulation of a-EJ pathways, the role of a-EJ in human disease, and the potential utility of a-EJ as a therapeutic target in cancer.

DNA double strand breaks (DSBs) can be generated by exposure to exogenous agents, such as ionizing radiation, endogenous agents such as reactive oxygen species generated aerobic metabolism or DNA metabolic processes, including DNA replication, meiosis and rearrangement of genes encoding immunoglobulins and T cell receptors. These are extremely dangerous lesions because the integrity of both strands of the DNA duplex is lost. In a cell with more than one DSB, it is important to rejoin the previously linked DNA ends otherwise a chromosomal translocation will be generated. Surprisingly, the predominant repair pathway in human cells that is often called either classic or canonical non-homologous end-joining relies upon DNA end-bridging mediated by protein-protein interactions involving DNA-dependnet protein kinase molecules to bring together DNA ends (1-3). In this review, we will refer to this pathway simply as non-homologous end-joining (NHEJ). While this could potentially join DNA ends from different chromosomes, the repair of DSBs by NHEJ usually results in the rejoining of previously linked DNA ends, possibly because the arrangement of chromatin in loops attached to a scaffold restricts the movement of DNA ends generated by a break in the loop. The majority of DSBs generated by ionizing radiation and oxygen free radicals have damaged and non-complementary termini that require processing prior to re-joining. This processing frequently results in either the loss or the addition of a few nucleotides at the break site (1,2). Thus, although NHEJ usually rejoins previously linked DNA ends, the repair of DSBs by this pathway is frequently mutagenic. For more details about the NHEJ pathway, see the accompanying review by Pannunzio et al.

While DSBs are repaired by NHEJ throughout the cell cycle, a recombinational repair pathway operates during the S and G2 phases of the cell cycle when an intact sister chromatid is available to guide the error-free repair of DSBs
(4,5). The initial steps of this pathway involve resection of the 5' ends of the DSBs followed by strand invasion into the adjacent intact sister chromatid, generating a D loop structure by strand exchange (5,6). For more details about the homologous recombination (HR) pathway, see the accompanying review by Heyer et al. In this review, we focus on minor DSB repair pathways that are genetically distinct from HR and NHEJ that we will refer to collectively as alternative end joining (a-EJ) pathways. These pathways do share factors with and/or utilize similar mechanisms to the major DSB repair pathways. All the a-EJ pathways, like HR, are initiated by end resection (Fig. 1) and involve some, if not all, of the factors that constitute the HR end resection machinery (1,6,7). The a-EJ pathways also share similarities with NHEJ in that the DNA ends to be joined are juxtaposed without using a homologous template as a guide. They do, however, utilize differing amounts of sequence homology (Fig.1) to align the DNA molecules (1,7). Although the a-EJ pathways make only a minor and poorly understood contribution to DSB repair in non-malignant cells, there is growing interest in these pathways as they generate large deletions, translocations and end-to-end chromosome fusions, genomic rearrangements that are frequently observed in cancer cells (8-10). Furthermore, they appear to be promising therapeutic targets in cancer cells with defects in either NHEJ or HR (9,11-14).

Overview of a-EJ in humans and its relationship with other pathways of DSB repair

A series of x-ray sensitive mutants of Chinese hamster ovary cell lines were used to identify human XRCC (X-ray cross-complementing) genes involved in the repair of DSBs both by HR and NHEJ (15,16). Around the same time, a number of labs described robust DNA end joining activities in extracts from mammalian cells but did not definitively link these activities to NHEJ factors (17,18). In a seminal paper, Bauman and West described end joining by a human cell extract that was dependent upon NHEJ factors but also noted that end joining activities that were independent of NHEJ could be detected in extracts prepared by different methods (19).

The initial genetic characterization of a-EJ pathways also occurred around the same time using the yeast Saccharomyces cerevisiae as a model eukaryote. In contrast to mammalian cells, HR is the predominant DSB repair pathway in yeast. Two minor DSB repair pathways, single strand annealing (SSA) and microhomology-mediated end joining (MMEJ) (20,21), were identified in HR-deficient yeast strains in addition to the NHEJ pathway (22). Both the SSA and MMEJ pathways are initiated by DNA end resection. In SSA, 5' to 3' end resection at both ends exposes single strand regions with complementary sequences of greater than 25 nucleotides that reside within tandem repeats (Fig. 1). The complementary sequences anneal, generating DNA duplex with non-complementary 3' single strand tails. These tails are removed, followed by gap filling synthesis and ligation. This pathway usually generates intrachromosomal deletions but may generate translocations through events involving repetitive elements on different chromosomes. In MMEJ, shorter regions of complementary sequence, ranging from 2 to 20 nucleotides that are called microhomologies, are frequently used to align DNA ends prior to gap filling and ligation. Like SSA, this pathway generates deletions but additional, non-templated nucleotides, may be added at the repair site (22).

It should be noted that the NHEJ pathway also utilizes microhomologies, such as those generated by restriction endonucleases, during end joining. While the complementary single strand overhangs generated by restriction endonucleases are usually accurately rejoined by the NHEJ pathway, microhomologies less than 4 nucleotides produced by limited nucleolytic processing and error-prone gap filling synthesis likely play a role in end alignment during the repair of DSBs with non-complementary ends by NHEJ, resulting in the characteristic small insertions and deletions (1). The ring-shaped Ku heterodimer initiates the repair of DSBs by NHEJ. This factor binds rapidly and stably to DSBs, preventing end degradation by the HR end resection machinery and limiting resection by other nuclease. It also serves as the platform for the assembly of the other NHEJ factors, including end processing factors, in a multiprotein complex (1,2,23). MMEJ and the other a-EJ pathways are distinct from NHEJ in that they are Ku-independent, require components of HR end resection machinery
and frequently involve longer tracts of microhomology. The competition between Ku and the end resection machinery for a DSB end determines whether that end will be repaired by NHEJ or channeled into the HR and occasionally the a-EJ pathways. Our current understanding of the mechanisms that determine DSB repair pathway choice is the focus of the accompanying review by Bunting et al.

The identification of PARP-1 and DNA ligase IIIα (LigIIIα), neither of which are present in S. cerevisiae, as participants in a-EJ in mammalian cells (Table 1), provided the first evidence that there are likely to be differences in the repertoire and mechanisms of a-EJ pathways between yeast and mammalian cells (24-26). The repair of DSBs by a-EJ, in particular MMEJ, is more evident in mammalian cells that are deficient in NHEJ (27-30). For example, class switch recombination that is normally dependent upon NHEJ factors occurs by an a-EJ pathway in the absence of a functional NHEJ pathway (27,30). Similarly, cells that are deficient in HR are more dependent upon a-EJ pathways for the repair of DSBs (9,11). In comparison to HR and NHEJ, the factors involved in a-EJ and the mechanisms by which they act together to process and repair DSBs are not well-defined. It is possible that the repair of DSBs by a-EJ that occurs in repair deficient cells may not be carried out by distinct pathways. Instead, the factors required to repair a DSB may be dictated by the nature of the defect in the NHEJ or HR pathway.

While it is evident that the MMEJ pathway serves as a back-up pathway in cells that are deficient in either NHEJ or HR, the joining of DSBs by MMEJ can be detected in cells that are proficient for both NHEJ and HR (31). The role(s) of the a-EJ pathways when the major DSB repair pathways are functional is poorly understood. A recent study showing that MMEJ activity is induced by ionizing radiation suggests that this pathway may be responsible for the repair of a subset of DSBs with damaged termini that render them refractory to repair by NHEJ (32). Since the end resection machinery is activated in S phase cells, it is possible that the SSA pathway acts to repair DSBs in S phase cells that occur in unreplicated DNA (7). In the following sections, we provide a brief overview of the mechanisms of the a-EJ pathways, SSA and MMEJ, that are shown schematically in Figures 2 and 3, respectively.

**End Resection (MRN/CtIP).** As mentioned above, the a-EJ pathways are similar to HR in that they are initiated by end resection (Fig. 1). For HR and a-EJ (Figs. 2 and 3), end resection is initiated by the Mre11/Rad50/Nbs1 (MRN) complex and CtIP (Table 1). Studies with the functionally homologous yeast proteins suggest that an initial 3’ single strand region is generated by CtIP-enhanced MRN endonuclease activity followed by 3’ to 5’ exonucleolytic digestion by MRN (6,33,34). This allows the loading of the more processive nucleases, either Exo1 or DNA2 that then generate long stretches of single strand DNA (6). Since PARP-1 is required for the rapid recruitment of MRN to DSBs (35), it is possible that the involvement of PARP-1 in a-EJ is due to its role in recruiting MRN for the initial phase of end resection. This, however, seems unlikely, since PARP inhibitors do not reduce the viability of cells with a functional HR pathway whereas if they disrupted MRN recruitment and end resection then they would also be expected to impact the repair of DSBs by HR.

While genetic studies have implicated MRN and CtIP in both SSA and MMEJ (29,31,36-40), the role of Exo1 and DNA2 is not clear. If there are defects in HR at the strand exchange or later stages, it seems likely that Exo1 and/or DNA2 will have been involved in generating the single strand ends. Functional redundancy between Exo1 and DNA2 may explain why DNA2, Exo1 and also BLM, which interacts with both Exo1 and DNA2, appear to be dispensable for MMEJ (6). Since more extensive DNA end resection is likely to be needed to expose the homologous repeats required for SSA, DSB repair by this pathway probably involves Exo1 and/or DNA2 (Fig. 2) whereas the more limited end resection by CtIP and MRN may be sufficient for at least some of the DSB repair events catalyzed by the other a-EJ pathways involving either a few nucleotides of microhomology (MMEJ) (Fig. 3) or no homology (EJ) (31).

At the present time, little is known about how the extent of end resection is controlled either during HR or a-EJ. Notably, the 3’ to 5’ exonuclease of Mre11, which functions as a dimer
within the MRN complex, is sensitive to the presence of other DNA ends with non-complementary ends enhancing degradation and complementary ends inhibiting degradation (41,42). When degradation by Mre11 exposes a short tract of sequence that is complementary to another DNA end, the Mre11 exonuclease appears to pause, presumably transiently stabilizing DNA end alignment via the microhomologies (41,42). The involvement of the endo- and, in particular, the exonuclease activity of Mre11 in MMEJ is supported by results from a recent study showing that the repair of a plasmid substrate by a MMEJ-proficient XRCC1 immunoprecipitate that contains MRN and CtIP was blocked by small molecule inhibitors of either the endo- or the exonuclease activities of Mre11 (32). Interestingly, the loss of WRN, a member of the RecQ helicase family that is defective in the prototypic premature ageing syndrome, Werner’s syndrome, increased the size of deletions generated during MMEJ event suggesting that WRN functions to suppress resection in MMEJ even though WRN also possesses nuclease activity (43).

Given the role of end resection in a-EJ, it was generally assumed that DSB repair via SSA, MMEJ and the other EJ pathway occurs predominantly in S and G2 phase cells when the end resection machineries are active. Despite the negative regulation of end resection in G1 cells, it is evident that the repair of DSBs by MMEJ also occurs in this phase of the cell cycle (44). Both CtIP and MRN appear to be responsible for the end resection in G1 cells with 53BP1 enhancing both end resection and MMEJ (44). Knockdown of 53BP1 in G1 cells resulted in about a 2-fold increase in ionizing radiation-induced γ-H2AX foci remaining 8 and 24 h after radiation, indicating that a significant fraction of ionizing radiation-induced DSBs are repaired by MMEJ in G1 cells (44). Furthermore, since inactivation of NHEJ resulted in increased repair of DSBs by MMEJ in G1 cells, it appears that NHEJ suppresses DSB repair by MMEJ in G1 cells (44). While phosphorylation of CtIP by cyclin-dependent protein kinases is critical for its role in initiating resection for HR in G2 cells, CtIP is phosphorylated in a DNA damage-inducible manner by Polo-like kinase 3 to activate CtIP/MRN-dependent resection in G1 cells (45,46). In accord with the evidence that there is competition between NHEJ and MMEJ in G1 cells (44), resection by CtIP/MRN in G1 cells inhibits DSB repair by NHEJ (45). It should be noted that there is also evidence that DSB repair in G1 cells occurs via a resection-dependent version of NHEJ rather than MMEJ in addition to resection-independent NHEJ (47). Further studies are needed to resolve these apparently contradictory findings.

DNA end bridging and alignment A critical step in all the a-EJ pathways is the juxtaposing of DNA ends. Since SSA likely requires extensive resection by Exo1 and/or DNA2 to expose the homologous repeats (Fig. 2 and Table 1), this repair pathway is probably most active during the S and G2 phases of the cell cycle. For post replication breaks, the ends are likely held in the same vicinity as a consequence of sister chromatid cohesion with the repair event generating intrachromosomal deletions whereas resection at DSBs in non-replicated DNA in cells could lead to translocations involving homologous repeats on different chromosomes (7). Initial genetic studies in yeast identified Rad52 as a key component of the SSA pathway with similar studies implicating mammalian Rad52 protein (20,48). Notably, Rad52 protein has a robust single annealing activity and is able to anneal complementary single strands that are coated with RPA (49,50). Together these studies suggest that, during SSA, Rad52 anneals complementary RPA coated-sequences exposed by end resection (Fig. 2 and Table 1).

For the other a-EJ pathways, different proteins have been suggested to play roles in the bridging of DNA ends and alignment via microhomologies (Table 1). PARP-1, which competes with Ku for binding to DSBs, appears to have end-bridging activity (24) although the mechanism by which PARP-1 mediates end-synapsis, in particular the contribution of poly(ADP-ribosylation), has not been elucidated. As noted previously, PARP-1 is involved in the rapid recruitment of the MRN complex to DNA ends (35). Both the yeast and human versions of this complex have robust end-bridging activity (51,52), suggesting that the MRN complex can simultaneously engage two DNA ends, compare sequences and transiently align the DNA ends via exposed microhomologies during the course of endo- and exo-nucleolytic digestion (41,42).
More recently, an A-family DNA polymerase, Pol 0, has been identified as a key factor in a-EJ (9,11). Pol 0 has a helicase-like domain at its N terminal that is separated from the C-terminal polymerase domain by a long, unstructured central region. Notably, Pol 0 displaces RPA from single strand DNA and also interacts with Rad51 and inhibits Rad51-dependent HR (9,11,53). Thus, Pol 0 appears to actively compete with the HR machinery for resected DNA ends. Furthermore, Pol 0 is capable of searching for and aligning microhomologies, thereby contributing to end bridging (54,55).

**Removal of non-homologous 3’ tails** During the repair of DSBs by SSA, the long 3’ tails are coated with RPA (7). Following Rad52-mediated annealing of homologous repeat sequences (Fig. 2), the non-complementary 3’ tails are removed by the DNA structure-specific endonuclease ERCC1/XPF (Table 1) that interacts with and is stimulated by Rad52 (56). Whilst it is likely the end-joining events catalyzed by the other a-EJ pathways will also involve removal of non-complementary 3’ tails (Fig. 1), the identity of the nuclease(s) involved has not been definitively established (Table 1), presumably reflecting redundancy among these enzymes.

**Gap filling DNA synthesis** It is likely that, after the removal of the non-homologous tails, the aligned DNA duplexes will contain gaps. The identity of the DNA polymerase(s) involved in gap filling during the repair of DSBs by SSA (7) and microhomology-independent EJ pathway (Fig. 1) has not been definitively established (Table 1). In contrast, there is compelling evidence that Pol 0 participates in the majority of DSBs repaired by MMEJ (9,11,55). Notably, Pol 0 has robust terminal transferase activity in addition to template-directed synthesis activity and so generates insertions with significant sequence diversity at repair sites (55,57,58). While Pol 0 is clearly a key MMEJ factor, there is currently no evidence of functional interactions between Pol 0 and other MMEJ factors.

**Ligation** The repair of DSBs by the a-EJ pathways is completed by a DNA ligase once ligatable termini have been generated by end processing. Among the DNA ligases encoded by the three mammalian LIG genes, DNA ligase IV appears to only function in NHEJ, leaving the DNA ligases encoded by the LIG1 and LIG3 genes as the candidate enzymes for a-EJ (2,59,60). Since *S. cerevisiae* lacks a homolog of the mammalian LIG3 gene (61), it is likely that Cdc9 DNA ligase, the functional homolog of human LigI, is the predominant DNA ligase in yeast EJ. While the contribution of the DNA ligases encoded by the mammalian LIG1 and LIG3 genes to SSA has not been established (Table 1), there is substantial evidence indicating that LigIIIα is the major DNA ligase in the MMEJ pathway (24,25,62,63). In the nucleus, LigIIIα forms a stable complex with XRCC1, a DNA repair protein that is often referred to as a scaffold protein because of its interactions with a large number of DNA repair proteins (64). Both LigIIα and XRCC1 preferentially interact with poly(ADP-ribosylated) PARP-1, interactions that underlie the recruitment of the LigIIα/XRCC1 to in vivo DNA single strand breaks (65,66). Although PARP inhibitors reduce the repair of DSBs by MMEJ, there was no reduction in the recruitment of XRCC1 to DSBs (12,32), indicating that the recruitment of LigIIα/XRCC1 is not dependent upon poly(ADP-ribosylated) PARP-1. Interestingly, LigIIα/XRCC1 also physically and functionally interacts with the MRN complex with these two complexes acting together to digest and join DNA duplexes with non-complementary termini utilizing internal sequence microhomologies (32,67). Thus, it is possible that this interaction directs the recruitment of LigIIα/XRCC1 to DSBs undergoing repair by MRN. In support of this idea, ionizing radiation induces increased association of XRCC1 with Mre11 and CtIP, co-localization of XRCC1 with Mre11 and increased MMEJ activity (32). These changes in the behavior of XRCC1 and MMEJ activity are dependent upon phosphorylation of XRCC1 by casein kinase 2 in response to ionizing radiation (32), suggesting that this phosphorylation event(s) enhance the interaction with the MRN complex. Interestingly, the N-terminal zinc finger of LigIIα, which is required for intermolecular ligation in vitro, is also required for LigIIα-dependent MMEJ (62,68-70).

In mouse cells, the NHEJ pathway acts to suppress formation of translocations by LigIIα-
dependent MMEJ in response to DSBs induced either by site-specific nucleases or ionizing radiation (10,62,63). This is consistent with studies implicating MMEJ in the formation of translocations due to aberrant class switch recombination in mice deficient in NHEJ (8,27,30). The observation in some studies that translocation formation was not dependent upon XRCC1 (63,71) was surprising given the role of this protein in maintaining the stability and activity of nuclear LigIII\(\alpha\) and other studies indicating that XRCC1 is a key component of the MMEJ pathway (32,72). While there may be sufficient residual nuclear LigIII\(\alpha\) for translocation formation in the absence of XRCC1 (72), it is also possible that, when nuclear LigIII\(\alpha\) is absent, translocation formation occurs by a LigI-dependent EJ pathway (62). Notably, these events do not appear to involve end alignment via microhomologies (62). At the present time, it is not known how LigI is recruited to the repair site and whether other LigI-interacting proteins such as PCNA and RFC are involved in this EJ pathway (Table 1).

**Role of A-EJ in genome instability and human disease**

The repair of DSBs by a-EJ is inherently mutagenic, potentially giving rise to chromosomal translocations as well as intra- and inter-chromosomal deletions and insertions (8,30). In addition, the MMEJ pathway also contributes to the formation of end-to-end chromosome fusions (9). Given the prevalence of these types of rearrangements in the genomes of cancer cells, there is significant interest in understanding the contribution of a-EJ to cancer formation and progression. Although the majority of studies of genome instability have focused on the nuclear genome, deletions within the circular mitochondrial genome that have been implicated in a wide variety of human diseases frequently occur between repeats and/or involve microhomologies at the repaired site (73-77). These observations suggest that a-EJ pathways may contribute to deleterious changes in mitochondrial DNA but our understanding of the repertoire and mechanisms of DNA repair pathways, in particular DSB repair pathways, operating in mitochondria is still very limited. While there is compelling evidence that LigII\(\alpha\) is the only DNA ligase in mitochondria (78-83) and mitochondrial extracts are capable of utilizing internal microhomologies to join DNA molecules with non-complementary ends (83), further work is needed to definitively establish the mitochondrial localization of other a-EJ factors, such as Pol \(\theta\) and Mre11, and their participation in the repair of DSBs by a-EJ in mitochondria (83,84).

As noted previously, a-EJ is more readily detectable in cells that are deficient in NHEJ (8,28,29). In mice, genetic inactivation of NHEJ results in an increase in the frequency of chromosomal translocations with evidence of microhomologies at many of the ligation sites, indicative of joining by MMEJ (10,62). As expected, mouse cells deficient in PARP-1, CtIP or LigIII\(\alpha\) exhibit a reduced overall frequency of chromosomal translocations and less use of microhomologies in the translocations that do occur (62). Furthermore, p53-null mice that are also deficient in NHEJ develop pro B-cell lymphomas arising as a consequence of chromosomal translocations between the \(\text{IgH}\) and the \(c\)-\textit{myc} loci with the repair junctions characterized by insertions, deletions and microhomology (85,86). Together these results indicate that, in the mouse, the majority of translocations in lymphoid cells likely occur as a result of the joining of DSBs generated by V(D)J recombination with DSBs generated by activation-induced cytidine deaminase and reactive oxygen species at other chromosomal fragile zones (87) by MMEJ whereas NHEJ prevents tumor incidence by suppressing translocation formation. Although there is compelling evidence indicating that Pol \(\theta\) is an important contributor to MMEJ (9,11,55), there are, however, contradictory reports as to whether Pol \(\theta\)-dependent MMEJ enhances or prevents chromosomal translocations (9,11,55,58). As noted above, end-to-end chromosome fusions are generated by Pol theta-dependent MMEJ (9). While this activity is normally suppressed by protective protein complexes at telomere ends and NHEJ (9), the end-to-end fusion of chromosomes by Pol \(\theta\)-dependent MMEJ may occur in response to telomere shortening during cancer development.

In contrast to mouse cells (10,62), the majority of translocations in human cells appear to arise as a consequence of the repair of DSBs by NHEJ (88). It is possible that differences in the relative contributions of NHEJ and A-EJ pathways...
to DSB repair between mouse and human cells underlies this discrepancy. For example, there is evidence that NHEJ is much less active in mouse cells (89). It is likely that the frequency of translocation formation versus rejoining of previously linked ends is different for each of the a-EJ pathways and that the contribution of an individual a-EJ pathway to translocation formation will be determined by a combination of the tendency of that pathway to generate translocations and the contribution of that pathway to DSB repair. For example, if the repair of DSBs by NHEJ results in a low frequency of translocations but almost all DSBs repaired by NHEJ then this pathway will be responsible for most of the translocations produced. In addition, it is possible that the contribution of the individual a-EJ pathways to translocation formation may differ depending on whether the DSBs are generated by class switch recombination, site-specific endonucleases or other mechanisms.

**A-EJ as a therapeutic target in cancer**

The rational development of olaparib and other PARP inhibitors to selectively target HR-deficient breast and ovarian cancers in patients with an inherited predisposition for these tumors (90-92) has stimulated efforts to design similar synthetic lethal strategies for other cancers. As back-up pathways for the major DSB repair pathways, the a-EJ pathways are attractive potential therapeutic targets in cancers with defects in either HR and NHEJ since inhibiting a-EJ is unlikely to impact the growth and survival of normal tissues but should sensitize the cancer cells that are more dependent on a-EJ pathways to repair DSBs generated by endogenous and/or exogenous agents. In support of this model, reducing MMEJ by knockdown or knockout of Pol ε reduced the survival of both HR- and NHEJ-deficient cells (9,11,55). Furthermore, BRCA2-deficient tumor cells have higher steady state levels of Pol ε (11), suggesting that MMEJ is upregulated in these cells to compensate for the HR defect, and knockdown of Pol ε enhanced the killing of HR-deficient cells by PARP inhibitors (11), indicating that MMEJ enhances the survival of HR-deficient cells by repairing replication-induced DSBs. While MMEJ inhibitors are likely to enhance the efficacy of PARP inhibitors in HR-deficient tumors, there is a more urgent need to develop reliable biomarkers to identify HR-deficient sporadic tumors that are likely to respond to PARP inhibitors. Based on initial studies (11), Pol ε expression levels appear to be a promising indicator of HR status.

In contrast to HR, there is less evidence linking mutation of NHEJ genes with genome instability in tumor samples. There is, however, evidence of reduced expression of the NHEJ factors, Ku, Artemis and LigIV, and a compensatory increase in MMEJ factors, PARP1 and LigIIα, in tyrosine-kinase activated leukemias, breast cancer and neuroblastoma (12-14). Notably, expression of either BCR-ABL1 or FLT3-ITD in non-malignant myeloid cell lines induces expression of c-MYC that in turn enhances expression of the Lig3 and PARP1 genes by suppressing expression of the microRNAs, miR-150 and miR-22 (93). Furthermore, the extent of the change in the expression levels of the DSB factors increases in imatinib-resistant chronic myeloid leukemia (CML) cells (14). An increase in the steady state levels of PARP1 and LigIIα was also observed in derivatives of an estrogen-responsive breast cell line that had acquired resistance to either tamoxifen or an aromatase inhibitor (13).

The changes in steady state levels of the DSB repair proteins in the breast cancer and BCR-ABL1-expressing myeloid cell lines correlated with changes in the relative contribution of NHEJ and MMEJ to the repair of a transfected plasmid substrate (13,14). As expected, incubation of these cell lines with a PARP inhibitor and an inhibitor of DNA ligases I and III, reduced the fraction of plasmids repaired by MMEJ (13,14). The cancer cell lines with dysregulated expression of the DSB repair proteins exhibited sensitivity to the PARP and DNA ligase inhibitors as single agents and in combination (12-14). Knockdown of LigIIα expression had similar effects to the LigI/III inhibitor suggesting that the activity of the inhibitor is due to inhibition of LigIIα rather than LigI (14). The synergistic activity of the PARP and DNA ligase inhibitors observed in some cell lines (13) is difficult to reconcile with the inhibition of two proteins in the same repair pathway. A recent study showing that the DNA ligase inhibitor preferentially targets mitochondrial function in cancer cells (94) suggest that the synergy may be
due to effects on both mitochondrial DNA metabolism and nuclear DNA repair.

Analysis of the expression levels of a-EJ and NHEJ genes in neuroblastoma showed that high expression of PARP1, LIG3 and LIG1 and low expression of LIG4 correlated with reduced survival and higher stage disease (12). Furthermore, elevated expression of both LIG3 and PARP1 was detected by RT-PCR in bone marrow mononuclear cells from CML patients with imatinib-resistant and imatinib sensitive disease (14). Notably, increased sensitivity to the combination of PARP-1 and DNA ligase inhibitors was observed in cells with elevated expression of both LIG3 and PARP1 (14). Taken together, these results indicate that MMEJ is a promising therapeutic target in cancers with elevated expression of genes encoding key MMEJ factors and/or reduced expression of genes encoding NHEJ factors.

Concluding Comments

In contrast to the two major DSB repair pathways, HR and NHEJ, the protein participants in and the molecular mechanisms of the minor DSB pathways, known collectively as a-EJ, are poorly defined. It had been suggested that a-EJ events did not reflect the activity of distinct DSB repair pathways but instead represented the action of a group of factors whose participation was dictated by the nature of the defect in the NHEJ or HR pathway. This view was based upon the observations that the repair of DSBs by a-EJ was more evident in cells that are deficient in either of the major DSB repair pathways and that all of the factors initially implicated in a-EJ had major roles in other DNA repair pathways. The recent demonstration that the major cellular function of Pol 0 is in a-EJ and the emerging evidence that a-EJ pathways contribute to DSB repair, even when the major DSB repair pathways are active, provides strong evidence that there are distinct a-EJ pathways and that the a-EJ pathways do make contributions, albeit not well understood, to DSB repair and genome stability in cells with functional HR and NHEJ pathways.

The role of the a-EJ pathways in the formation of large genomic rearrangements, in particular translocations, that are characteristic of cancer cells is an active area of investigation. While it is evident that the a-EJ pathways as well as the NHEJ pathway are capable of contributing to this type of genome instability, there are apparently contradictory published findings. This may reflect differences in the utilization of DSB repair pathways between mice and humans and between different cell types. In addition, it is possible that the different assays used to detect genome rearrangements may produce different results. There is, however, compelling evidence that human cancer cells with defects in either the HR or NHEJ pathways are more dependent upon a-EJ pathways, in particular, MMEJ for DSB repair, providing a rationale for the development of therapeutic strategies that target the MMEJ pathway. Furthermore, it appears that elevated expression of MMEJ factors, such as PARP-1, LigIIIα and Pol 0, may serve as biomarkers for cancers with defects in the two major DSB repair pathways, thereby identifying the patient population whose disease is likely to respond to inhibitors of MMEJ.

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Conflicts of Interest: A. E. Tomkinson is a co-inventor on patents that cover the use of DNA ligase inhibitors as anti-cancer agents, and altered expression of DSB repair proteins as biomarkers of increased dependence upon MMEJ. A. Sallmyr has no competing interests.

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Table 1. Proteins involved in a-EJ pathways. For the proteins shown in bold, there is strong evidence identifying them as a key factor in the indicated EJ pathway.

| Protein   | SSA   | MMEJ       | EJ       |
|-----------|-------|------------|----------|
| MRN/CtIP  | RAD52 | PARP/MRN/Polθ | PARP/MRN |
| EXO1/DNA2|       |            |          |
| MRN/CtIP  | PARP/MRN/Polθ |            |          |
| EXO1/DNA2|       |            |          |

FIGURE LEGENDS:

**Figure 1. The role of DNA sequence homology in a-EJ pathways.** Resection of the 5’ strand at DSBs is the first common step of all the EJ pathways (a-EJ). Three distinct pathways, single strand annealing (SSA), microhomology-mediated end joining (MMEJ) and end-joining (EJ) are distinguished based on the amount of DNA sequence complementarity used to align DNA ends. SSA involves complementary repeat sequences more than 25 nucleotides in length whereas MMEJ involves shorter tracts of sequence homology, ranging from 2 to 20 nucleotides in length. There is also a third category of DSB repair events that either lack or have very little sequence homology at the repair site generated by a poorly defined EJ pathway.

**Figure 2. Repair of DSBs by the Single Strand Annealing Pathway.** 1) Introduction of a DSB break; 2) PARP-1 (PARP) mediates the rapid recruitment of MRN and CtIP to the DSB end. CtIP enhances the MRN endonuclease activity resulting in an internal single strand break within the 5’ strand. The short single strand fragment at the DSB end is then degraded by the MRN exonuclease activity; 3) The resultant single strand...
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Region, which is rapidly bound by RPA, serves as the binding site for one of the processive 5’ to 3’ exonucleases, either Exo1 or DNA2; 4) The resultant long range resection by Exo1 or DNA2 exposes complementary single strand regions, greater than 25 nucleotides in length; 5) Rad52 interacts with the RPA-coated single strands and anneals the complementary regions, aligning the DNA ends and exposing non-homologous 3’ single strand tails; 6) The single strand tails are removed by ERCC1/XPF, a DNA structure-specific endonuclease that cleaves the 3’ strand at duplex/single strand junctions; 7) After any gaps are filled, both strands are ligated to generate an intact duplex that is missing one of the repeats and the DNA region between the repeats. The DNA polymerases and DNA ligases involved in the last steps of SSA have not been identified.

Figure 3. Repair of DSBs by the Microhomology-Mediated End-Joining Pathway. 1) Introduction of a DSB break; 2) PARP-1 (PARP) mediates the rapid recruitment of MRN and CtIP to the DSB end. CtIP enhances the MRN endonuclease activity resulting in an internal single strand break within the 5’ strand. The short single strand fragment at the DSB end is then degraded by the MRN exonuclease activity; 3) Short regions of sequence complementarity, ranging from 2 to 20 nucleotides, are exposed within the RPA-coated single strand regions; 4) The DNA ends are transiently aligned via the short microhomologies. PARP-1, MRN and Pol θ have each been implicated in the end alignment. It is likely that a similar, possibly MRN independent process of end alignment also occurs between ends that are being resected by the long-range exonucleases, Exo1 and DNA2 (see Fig 2, step 3); 5) Non-homologous 3’ tails are removed prior to error-prone gap-filling DNA synthesis by Pol θ. It is assumed that several functionally redundant nucleases will participate in end processing; 6) Both strands are ligated by the LigIIIα/XRCC1 complex (LigIII/XRCC1); 7) The DNA duplexes generated by MMEJ are characterized by deletions and the presence of sequence microhomologies at the repair site.

Fig. 1
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Fig. 2

1) 
2) 
3) 
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6) 
7)
Repair of DNA double-strand breaks by mammalian alternative end-joining pathways
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