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The Role of Error-Prone Alternative Non-Homologous End-Joining in Genomic Instability in Cancer

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1. Introduction

To maintain the integrity of the genome, cells have evolved a complex set of pathways that function in response to DNA damage. Components of this response include *(i)* cell cycle checkpoints that prevent damaged DNA from being replicated, *(ii)* induction of programmed cell death to prevent the transmission of potentially mutagenic genetic changes and *(iii)* DNA repair pathways that remove various types of DNA lesions such as single base lesions, single strand breaks (SSB)s or double strand breaks (DSB)s.

DSBs are considered the most lethal form of DNA damage because, unlike almost any other types of DNA damage that have an intact undamaged template strand to guide the repair, the integrity of both strands of the duplex is lost (Khanna and Jackson, 2001). DSBs can be induced by environmental factors such as ionizing radiation, ultraviolet light, therapeutic treatment but also occur as a consequence of specific physiological processes such as DNA replication, the V(D)J recombination in B and T-lymphocytes or the immunoglobulin class switch recombination (CSR) within immunoglobulin variable domains in B-lymphocytes occurring during the development and maturation of the immune system (Ferguson and Alt, 2001, Revy et al., 2005). In order to maintain the integrity of the DNA information, cells recruit stringent DSB repair machinery to ensure the efficient repair of various types of DNA damage. Thus, failure to properly repair the DSBs may cause chromosomal abnormalities, which in turn, may lead to genomic instability and predispose the cells to malignant transformation. Moreover, the importance of DNA repair in protecting against DSB-induced genomic instability is suggested by the increased incidence of cancer in autosomal recessive DNA repair deficient human syndromes, such as BRCA1/2 deficient breast cancers (Futreal et al., 1994). Thus, since genomic instability is a common characteristic of both inherited and sporadic forms of cancer cells, it is likely that abnormalities in DNA repair contribute to the development and progression of sporadic cancers (Khanna and Jackson, 2001).

DSBs can be repaired by two major pathways, homology–directed repair (HR) and non-homologous end-joining (NHEJ) (Helleday et al., 2007). HR is active during the late S and G2 phases of the cell cycle and uses the intact sister chromatid as the template for repair

(Khanna and Jackson, 2001, Hartlerode and Scully, 2009). This pathway is a highly efficacious and error-free form of repair and is mainly responsible for the repair of DSBs caused by stalled/or collapsed replication forks induced for example by chemotherapeutic agents that abrogate DNA replication (Keller et al., 2001). HR mechanisms and their implication in genomic stability are reviewed in detail in Khanna and Jackson, 2001, Helleday et al., 2007, Hartlerode and Scully, 2009.

NHEJ repairs DSBs quite differently from HR by joining DNA ends directly. This form of repair is independent of extensive DNA sequence homology, and therefore errors can be introduced during the processing and joining of non-compatible DNA ends (Khanna and Jackson, 2001, Lieber, 2008, Hartlerode and Scully, 2009). NHEJ occurs throughout the cell cycle and is the major DSB repair pathway in G0, G1 and early S phase. NHEJ is the preferential pathway for repair of DSBs in mammalian cells (Lieber et al., 2003, Lieber, 2008).

Here, we describe the mechanism(s) and the role(s) of the error-prone NHEJ pathway in the maintenance of genomic instability in cancer and discuss how targeting NHEJ is a promising therapeutic strategy in cancer.

2. Error-prone NHEJ pathway: Mechanisms and properties in normal and cancer cells

Classical or C-NHEJ contributes to the repair of DSBs caused by endogenous and exogenous DNA damaging agents and also plays an important role in the repair of programmed DSBs in normal mammalian cells, made during V(D)J or CSR (Lieber et al., 2006). In addition, evidence now exists for an alternative version of NHEJ (ALT-NHEJ) (Nussenzweig and Nussenzweig, 2007) that exists at low levels in normal cells (Sallmyr et al., 2008b) and is enhanced in the absence of C-NHEJ. Here, we discuss the mechanisms and properties of C-NHEJ and ALT-NHEJ in normal and cancer cells.

2.1 The C-NHEJ pathway

There appears to be two phases of C-NHEJ: a rapid phase and a slower phase (Riballo et al., 2004). The rapid phase will repair most of the simple lesions which do not require any type of processing. In contrast, the slower phase of NHEJ reflects both the repair of *(i)* DSBs that occur in condensed chromatin and *(ii)* more complex DSBs that require processing before ligation (Riballo et al., 2004, Goodarzi et al., 2008).

The C-NHEJ pathway is initiated by the Ku70/Ku86 heterodimer also called Ku, a ring shaped complex that binds DSBs (Walker et al., 2001). This leads to the recruitment of the catalytic subunit of DNA dependent protein kinase (DNA PKcs) (Mimori and Hardin, 1986, Falzon et al., 1993, Gottlieb and Jackson, 1993) to form the activated DNA PK (Calsou et al., 1999, Singleton et al., 1999). The kinase activity of DNA PK is critical for C-NHEJ (Lees-Miller et al., 1990). DNA PK also phosphorylates other proteins, such as Artemis, which binds to DNA PKcs (Ma et al., 2002), activating its endonuclease activity at both 3′ and 5′ overhangs. The physical juxtaposition of DNA ends involves interactions between DNA-bound DNA PKcs molecules (Yaneva et al., 1997, DeFazio et al., 2002). If DNA ends can be directly ligated then the repair only requires ligation by XLF/DNA ligase IV/XRCC4, after interaction with DNA PK (Ahnesorg et al., 2006, Buck et al., 2006). However, a large fraction of DSBs generated by agents such as, ionizing radiation, are not directly ligatable, and require additional processing (Chen et al., 2000, Lobrich and Jeggo, 2005). Many proteins are

involved in processing these DNA ends, including polynucleotide kinase (PNK) which interacts with XRCC4 (Chappell et al., 2002), the nucleases Flap endonuclease-1 (FEN-1) (Wu et al., 1999) and Artemis (Chen et al., 2000), and the Polymerase X family members, Pol μ and λ (Ma et al., 2004). As a consequence of these processing reactions, the joining of DSBs by C-NHEJ often results in the loss or addition of a few nucleotides and the presence of short complementary sequences, microhomologies, at the break site that presumably contribute to the alignment of the DNA ends (Roth et al., 1985, Roth and Wilson, 1986).
A schematic of the C-NHEJ is presented in **Figure 1**.

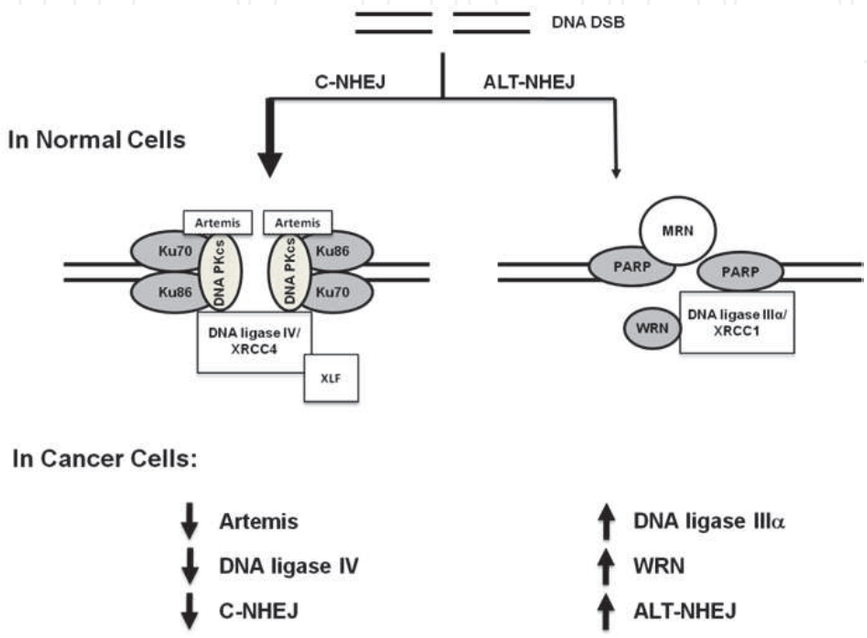

Fig. 1. In normal cells (upper panel), ALT-NHEJ pathway is a minor DSB repair pathway compared with C-NHEJ. In cancer cells (lower panel), the steady state levels of key C-NHEJ proteins are reduced whereas the steady state levels of key ALT-NHEJ are increased. This results in increased activity of the ALT-NHEJ pathway and reduced activity of the C-NHEJ pathway. Figure modified from Rassool and Tomkinson, 2010.

2.2 The ALT-NHEJ pathway

There are several lines of evidence for an alternative or back-up version of NHEJ that is enhanced in the absence of C-NHEJ (Riballo et al., 2004, Wang et al., 2006, Nussenzweig and Nussenzweig, 2007) **(Figure 1)**. While these studies have begun to define more precisely the characteristics, mechanisms, regulation and roles of ALT-NHEJ in the development and maintenance of cancer, much of this pathway(s) remains to be elucidated. In the next section, the current state of our knowledge of ALT-NHEJ will be discussed.

2.2.1 Key signatures of ALT-NHEJ

The key features of the ALT-NHEJ pathway are that the repair junctions are characterized by larger deletions, insertions, and longer tracts of microhomology compared with those generated by C-NHEJ, and a much higher frequency of chromosomal translocations (Nussenzweig and Nussenzweig, 2007).

2.2.1.1 Microhomologies

Mechanistically, 3′ single stranded overhangs containing longer tracts of microhomology are used to mediate ALT-NHEJ (Corneo et al., 2007, Yan et al., 2007, Bennardo et al., 2008, Deriano et al., 2009, Dinkelmann et al., 2009, Rass et al., 2009, Xie et al., 2009). This generally involves the loss of the intervening DNA sequences between the microhomology containing regions, resulting in larger DNA deletions. The regions of microhomology always reside at the precise site of repair and can be used as a marker to define these repair events. Moreover, while, ALT-NHEJ is associated with the generation of 3′ single stranded overhang at the sites of DSBs, the presence of the DNA end-processing factor CtlP, appears to be required for microhomology-mediated joins upon depletion of the C-NHEJ component Ku70 (Lee-Theilen et al., 2011). Notably, microhomology sequences suggestive of ALT-NHEJ have been found at the recombination junctions of radiation-induced genomic rearrangements (Morris and Thacker, 1993, Nohmi et al., 1999) implying that radiation-induced DSBs can be repaired by ALT-NHEJ. Moreover, microhomologies are frequently detected at the breakpoints of chromosomal deletions and translocations in human cancer cells (Canning and Dryja, 1989, Dryja et al., 1989, Smanik et al., 1995, Wiemels and Greaves, 1999).

2.2.1.2 Translocation frequency

Several groups have observed that in the absence of C-NHEJ proteins, chromosomal translocations occur with increased frequency (Boboila et al., 2010b, Simsek and Jasin, 2010). These authors thus suggested that C-NHEJ suppresses chromosomal translocations. An alternative explanation for the increase in translocation frequency when C-NHEJ is absent, is that end-joining may be inefficient due to missing or mutant NHEJ components, and this may lead to the accumulation of multiple unrepaired DSBs. There is evidence that the repair kinetics of ALT-NHEJ is slower than that of C-NHEJ, in that end-joining assays performed in cells lacking DNA ligase IV are about 10 times slower than in cells proficient for C-NHEJ (Yan et al., 2007, Han and Yu, 2008). Thus, slowed NHEJ would be expected to increase the time of overlap during which two breaks would remain unrepaired, thereby increasing the chance of translocation events (Lieber, 2010).

Recent studies have suggested that oncogenes critical in the pathogenesis of leukemias directly or indirectly down regulate steady state levels of key C-NHEJ proteins, and in concert, upregulate key ALT-NHEJ proteins, leading to an increase in the frequency of deletions and translocations, which likely drive genomic instability, disease progression or resistance to treatment (Chen et al., 2008, Sallmyr et al., 2008b, Fan et al., 2010, Li et al., 2011).

2.2.2 Components involved in ALT-NHEJ

The presence or absence of Ku at the DSB dictate whether repair occurs *via* C-NHEJ or ALT-NHEJ, respectively (Fattah et al., 2010, Cheng et al., 2011). Several DNA repair proteins have been implicated in ALT-NHEJ repair. These include, DNA ligase IIIα/XRCC1, poly(ADP) ribose polymerase-1 (PARP-1), the MRN complex (Mre11/Rad50/Nbs1), WRN and CtlP (Audebert et al., 2004, Wang et al., 2005, Wang et al., 2006, Rass et al., 2009, Robert et al., 2009, Xie et al., 2009, Lee-Theilen et al., 2011, Cheng et al., 2011, Zhang and Jasin, 2011).

Given that ALT-NHEJ is initiated by resected DNA ends, the question arises, which factors can bind resected DSBs to start this repair process? Recent work identified PARP-1 as an

additional potential contributor to ALT-NHEJ (Audebert et al., 2004). PARP-1 recognizes DNA strand interruptions *in vivo* and triggers its own modification as well as that of other proteins by the sequential addition of ADP-ribose to form polymers. PARP-1 intervenes in base excision and single strand annealing (SSA) and now also operates in ALT-NHEJ (Audebert et al., 2004, Wang et al., 2006). While its role in ALT-NHEJ remains to be clearly elucidated, Wang et al. showed that PARP-1 binds to DNA ends in direct competition with Ku (Wang et al., 2006). When essential components of C-NHEJ are absent, PARP-1 is recruited for DSB repair, particularly in the absence of Ku proteins (Wang et al., 2006, Cheng et al., 2011).

The next question that arises is, which factor(s) is involved in the final joining reaction of ALT-NHEJ? Several studies implicate DNA ligase IIIα in ALT-NHEJ (Audebert et al., 2004, Wang et al., 2005, Haber, 2008). For example, using extract fractionation studies, Wang et al., showed that the majority of DNA end joining activity in extracts of HeLa cells could be attributed to DNA ligase IIIα (Wang et al., 2005). In addition, immunodepletion of DNA ligase IIIα from cell extracts caused loss of activity that could be recovered by the addition of the joining activity contributed by the purified enzyme. These experiments also ruled out a significant contribution to the end joining activity by DNA ligase I and DNA ligase IV. Furthermore, Wang et al., also addressed this question using RNA interference to investigate the requirements for DNA ligase IIIα and DNA ligase IV in the repair of DSBs (Wang et al., 2005). *In vivo* plasmid assays showed that DNA ligase IV-deficient mouse embryonic fibroblasts (MEFs) retained significant DNA end joining activity that could be reduced by up to 80% in cells knocked down for DNA ligase IIIα using RNAi (Wang et al., 2005). These *in vivo* observations are in line with DNA ligase IIIα being a candidate component for ALT-NHEJ. Other studies have implicated additional factors in ALT-NHEJ, such as PNK, FEN-1 (Gottlich et al., 1998, Wang et al., 2003, Audebert et al., 2004, Wang et al., 2006), and it is expected that additional factors will also be identified in the future **(Figure 1)**.

2.2.3 Where ALT-NHEJ fits into the hierarchy of DSB repair?

While there is strong evidence that ALT-NHEJ is enhanced in cells that are defective for C-NHEJ, the question of where ALT-NHEJ fits into the hierarchy of DSB repair with respect to the cell cycle, and what would be the consequences of this repair at the genomic level, are still relatively unclear (**Figure 2**).

2.2.3.1 ALT-NHEJ and cell cycle

While it is well documented that HR is efficiently carried out only in the late S and G2 phases of the cell cycle using the newly synthesized sister chromatid, whereas C-NHEJ is the major DSB repair pathway in G0, G1 and early S phase (Lieber et al., 2003, Lieber et al., 2006), recent studies suggest that ALT-NHEJ may also be cell cycle dependent. During DNA replication, the newly replicated chromatids are held together by cohesin and this sister chromatid cohesion is maintained until mitosis. When a DSB occurs, the intact sister chromatid is preferentially used to repair the DSB by HR. If HR is defective, as it is demonstrated in BRCA 1/2 deficient cells, DSB is likely to be repaired by the following error-prone pathways (Tutt et al., 2001, Venkitaraman, 2001): *(i)* SSA that generates intrachromosomal deletions between repeated sequences, *(ii)* C-NHEJ pathway that generates small intrachromosomal deletions and insertions, and *(iii)* ALT-NHEJ pathway that generates larger deletions and chromosomal translocations. One of the roles of the DNA PK complex assembled on the DNA end is to

protect the DNA end from resection (Huertas, 2010). If C-NHEJ is defective, it is likely that end resection will occur (**Figure 2**). While the above hypothetical scenarios for error-prone repair of DSBs are envisioned, recent studies suggest that ALT-NHEJ may occur more frequently in G2. Mladenov and Iliakis enquired whether ALT-NHEJ was cell cycle dependent. In this study, MEFs with defects in C-NHEJ and/or HR were irradiated, G1 and G2 cells were isolated by cell sorting, and repair was examined by using pulse field gel electrophoresis (Mladenov and Iliakis, 2011). They found that wild-type and HR defective (*Rad54*$^{-/-}$) MEFs repaired DSBs with similar efficiency in G1 and G2 phases. In contrast, C-NHEJ defective (*DNA ligase IV*$^{-/-}$, *DNA PKcs*$^{-/-}$, and *Ku70*$^{-/-}$) MEFs showed a more pronounced repair defect in G1 phase than in G2 phase. Importantly, *DNA ligase IV*$^{-/-}$/*Rad54*$^{-/-}$ MEFs repaired DSBs as efficiently as *DNA ligase IV*$^{-/-}$ MEFs in G2 suggesting that the increased repair efficiency in G2 phase relies on the enhanced function of ALT-NHEJ rather than on HR. Furthermore, *in vivo* and *in vitro* plasmid end joining assays confirmed an enhanced function of ALT-NHEJ in G2 phase (Mladenov and Iliakis, 2011). Additional studies along the same lines using mutant Chinese hamster cells with defects in the DNA PKcs, Ku86 or XRCC4 components of C-NHEJ, or in the XRCC2 and XRCC3 components of HR confirmed these observations (Wu et al., 2008). Wild-type cells and mutants of HR repaired DSBs with similar efficiency in G1 and G2 phases. Mutants of C-NHEJ, showed more pronounced repair in G2 phase than in G1. These results in aggregate demonstrate a new and potentially important cell cycle regulation of ALT-NHEJ and generate a framework to investigate the mechanistic basis of HR contribution to DSB repair and its possible interactions with ALT-NHEJ.

Yet another study by Shibata et al., also examined the regulation of repair pathway usage at DSBs in G2 (Shibata et al., 2011). They identified the speed of DSB repair as a major component influencing repair pathway usage showing that DNA damage and chromatin complexity are factors influencing DSB repair rate and pathway choice. They found that loss of C-NHEJ proteins slowed DSB repair allowing increased resection. In contrast, loss of HR does not impair repair by C-NHEJ although CtlP-dependent end-resection precludes C-NHEJ usage. These data suggest that C-NHEJ initially attempts the repair of DSBs and, if rapid rejoining does not ensue, then resection occurs promoting repair by HR using the homologous chromosome as template, but this may result in loss of heterozygosity (LOH). It is likely that if repair does not occur by HR, DNA ends will be repaired by error-prone pathways, such as SSA and ALT-NHEJ, pathways that require end-resected DSBs (Shibata et al., 2011) (**Figure 2**).

2.2.3.2 Factors regulating ALT-NHEJ

Unlike C-NHEJ, the mechanism(s) for regulation of ALT-NHEJ and the factors involved in this repair pathway(s) are not clearly understood. The presence of Ku proteins appear to determine whether DSBs are repaired by C-NHEJ *vs*. ALT-NHEJ (Bennardo et al., 2008, Fattah et al., 2010, Cheng et al., 2011). Fattah et al., utilized an end-joining assay in isogenic human colon carcinoma cell lines and human somatic HCT116 with targeted deletions of the key C-NHEJ factors (Ku, DNA PKcs, XLF, and DNA ligase IV). The end-joining assay was a plasmid based repair assay of a DSB made within reporter plasmid pEGFP-Pem1-Ad2 and reconstitution of green fluorescent protein (GFP). They found that absence of key C-NHEJ factors resulted in cell lines that were profoundly impaired in DSB repair activity. Unexpectedly, Ku86-deleted cells showed wild-type levels of DNA DSB repair activity but the events were mainly repaired by microhomology joining. Using siRNA technology, ALT-

NHEJ repair activity could also be efficiently activated in *DNA ligase IV*$^{-/-}$ and *DNA PKcs*$^{-/-}$ cells by subsequently reducing the level of Ku70. Recently, Cheng et al., demonstrated that Ku is the main factor preventing PARP-1 and MRN mobilization to the site of DSBs (Cheng et al., 2011). These studies demonstrate that Ku proteins are the critical C-NHEJ factors that regulate DSB repair pathway choice. Similarly, studies of Bennardo et al., compared the genetic requirements for ALT-NHEJ, using a series of chromosome integrated reporters to monitor repair of DSBs by the I-SceI endonuclease in mouse embryonic stem (ES) cells and the HEK293 cell line (Bennardo et al., 2008). Each individual reporter was designed such that repair of I-SceI-induced DSBs by a specific pathway restored a GFP expression cassette. Such repair was then scored in individual cells as green fluorescence using flow cytometric analysis. They found that the *Ku70*$^{-/-}$ cells exhibited a 4-fold increase in the restoration of the GFP^{+} gene over wild-type cells, and that this increase was reversed by co-transfection of a Ku70 expression vector (Bennardo et al., 2008). Thus, the ALT-NHEJ repair events appeared not only to be Ku-independent, but also appear to be inhibited by Ku proteins (Bennardo et al., 2008, Cheng et al., 2011). Bennardo and Stark have also highlighted the importance of the presence of ataxia telangiectasia-mutated (ATM) in matching correct DNA ends during end-joining and preventing the joining of multiple chromosome ends that can lead to chromosomal translocation and genomic instability (Bennardo and Stark, 2010). They found that genetic or chemical disruption of ATM caused a substantial increase in incorrect end joining (Distal-EJ), but not correct end joining (Proximal-EJ). Moreover, the increase in Distal-EJ caused by ATM disruption was dependent on the presence of C-NHEJ factors, specifically DNA PKcs, XRCC4, and XLF. Thus, these authors concluded that ATM is important to limit incorrect end utilization during C-NHEJ. In yet another study, Zha et al. showed that ATM and XLF have fundamental roles in processing and joining DNA ends during V(D)J recombination, but that these roles were masked by functional redundancies. They found that combined deficiency of ATM and XLF nearly blocked mouse lymphocyte development due to an inability to process and join chromosomal V(D)J recombination DSB intermediates. Combined XLF and ATM deficiency also severely impaired C-NHEJ, but not ALT-NHEJ, during CSR. Redundant ATM and XLF functions in C-NHEJ appeared to be mediated by ATM kinase activity and are not required for extra-chromosomal V(D)J recombination, indicating a role for chromatin-associated ATM substrates. These authors also found a role for H2AX, protein involved in the recruitment of DNA repair factors to nuclear foci after DSBs (Rogakou et al., 1998). Conditional H2AX inactivation in XLF-deficient pro-B lines leads to V(D)J recombination defects associated with marked degradation of unjoined V(D)J ends, revealing that H2AX also has a role in the repair process (Zha et al., 2011).

Mechanistically, it is believed that during ALT-NHEJ both broken ends are resected to generate 3′-single-stranded overhangs (Huertas, 2010). Given that Ku-deficiency can lead to elevated DSB end-processing, these results raise the possibility that ALT-NHEJ, SSA and HR share end-processing as a common intermediate. Thus, Bennardo et al., determined also whether end-resecting factor CtlP is important for ALT-NHEJ, by performing siRNA knock-down of CtlP in HEK293 cell lines with integrated GFP reporter plasmids and stable expression of the inducible I-SceI protein and examined ALT-NHEJ repair in CtlP-depleted cells *vs*. control cells. They observed that ALT-NHEJ was significantly reduced in CtlP-depleted cells suggesting that CtlP-mediated DSB end-processing promotes ALT-NHEJ but also SSA and HR (Bennardo et al., 2008). Interestingly, disrupting RAD51 and RAD52 expression decrease HR and SSA activity respectively without perturbing ALT-NHEJ repair

(Bennardo et al., 2008). In recent studies, Zhang and Jasin, showed that depletion of CtlP, resulted in a substantial decrease in the chromosomal translocation frequency in mouse cells and a significantly lower usage of microhomology at the translocation breakpoint junctions. This suggests that CtlP-mediated ALT-NHEJ has a primary role in translocation formation (Zhang and Jasin, 2011).

Several studies have also implicated the MRN complex in ALT-NHEJ repair (Rass et al., 2009, Xie et al., 2009). Recent studies examined the role of the nuclease MRE11 in CSR. They showed that loss of the nuclease MRE11 resulted in milder defects, compared with loss of the whole MRN complex. This suggested that the MRN complex performed activities in end-joining, in addition to the nuclease activity of MRE11. Studies employing atomic force microscopy have visualized the MRN complex bridging DNA at distances of 1200 angstroms (Moreno-Herrero et al., 2005). Thus, MRN may perform bridging functions that may be particularly suited for CSR. In addition, since chromosomal translocations are frequently observed in ALT-NHEJ, the MRN complex may also play a role in end-bridging of distant DSBs, resulting in chromosomal rearrangements (**Figure 2**).

A schematic representation of the regulation and the hierarchy of the DSB repair pathways is presented in **Figure 2**.

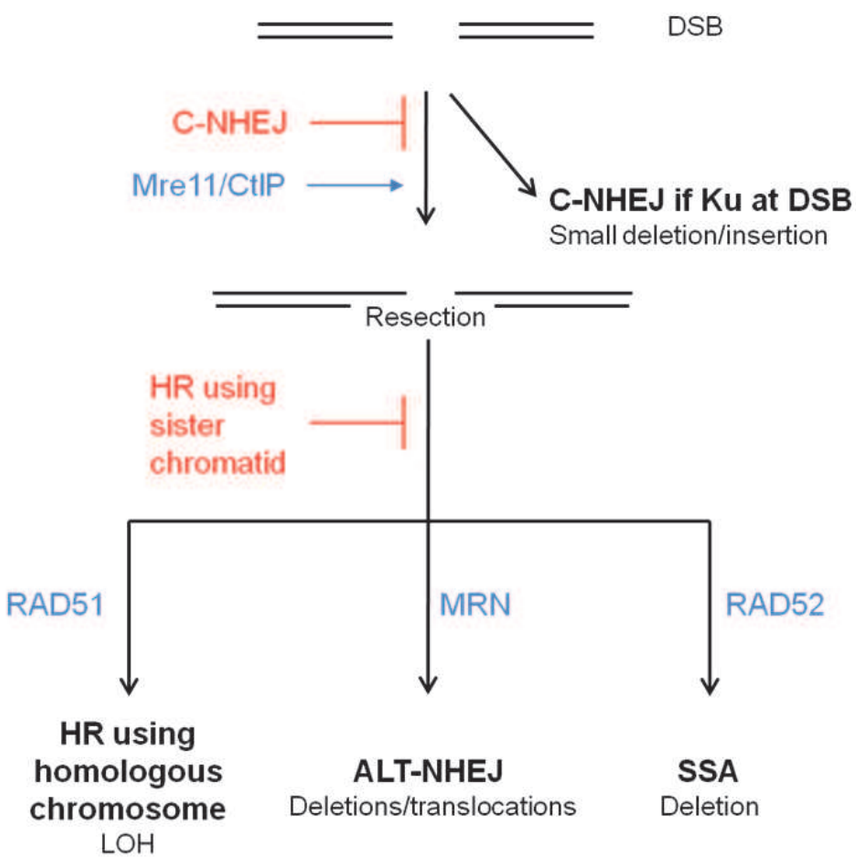

Fig. 2. The majority of the DSBs are repaired by C-NHEJ. If this pathway is inactive, DSBs can be repaired by HR using the homologous chromosome as template or by SSA or ALT-NHEJ. Positive regulators of specific stage as described in text are represented in blue while defective pathways are represented in red.

2.2.4 ALT-NHEJ at dysfunctional telomeres

Mammalian telomeres are regions of repetitive DNA sequences at the ends of chromosomes, which protect them from e or from fusion with neighbouring chromosomes. Critically shortened telomeres are recognized as DSBs and are highly susceptible to be repaired by HR or NHEJ pathways (Palm et al., 2009, Rai et al., 2010). However, unequal exchange of telomeric sequences by HR or misrepair by C- and/or ALT-NHEJ, can lead to loss of cell viability or can result in genomic instability and cancer. In mammals, telomeres form single-stranded G-rich overhangs that associate with and are protected by shelterin, a core complex of telomere-binding proteins that includes the double-stranded DNA-binding proteins TRF1 and TRF2 and protection of telomeres 1 (POT1a/b) that interacts with its binding partner TPP1 to protect them from resection, recombination and alteration (Palm et al., 2009). Telomeres are maintained by the enzyme telomerase, which is limited in human somatic cells, resulting in progressive telomere shortening. Celli et al., showed that Ku and TRF2 repress HR and represent an important aspect of telomere protection (Celli et al., 2006). Recent evidence suggests that dysfunctional telomeres that can no longer exert end-protective functions are recognized as DSBs by the DNA damage repair pathway. Thus, removal of TRF2 with retrovirus-mediated shTrf2, resulted in end-to-end chromosome fusions mediated by the C-NHEJ pathway (Rai et al., 2010). In addition, the data of Deng et al., indicated a critical role for the MRN complex in sensing these dysfunctional telomeres. They showed that in the absence of TRF2, MRE11 nuclease activity removes the 3' telomeric overhang to promote chromosome fusions. MRE11 can also protect newly replicated leading strand telomeres from NHEJ by promoting 5' strand resection to generate POT1a-TPP1-bound 3' overhangs (Deng et al., 2009). Rai et al. used also MEFs in which specific components of the C-NHEJ had been deleted to determine how dysfunctional telomeres are joined together (Rai et al., 2010). They showed that DSB marker 53BP1 (Schultz et al., 2000, Anderson et al., 2001) was necessary for end to end fusion in TRF2 deficient MEFs. Surprisingly, they showed that removal of *Tpp1-Pot1a/b* from *53BP1*$^{-/-}$ MEFs or *DNA ligase IV*$^{-/-}$ MEFs resulted in robust end to end fusions. They also examined chromosome fusion in MEFs from telomerase knock-out cells that generate naturally shortened telomeres, and which had also been knocked out for *53BP1*$^{-/-}$. Lymphomas derived from these mice demonstrated an increase in the number of fused chromosomes. These data suggested that fusion of naturally shortened telomeres do not require 53BP1 and occur through mechanisms independent of C-NHEJ. They concluded that telomeres engage distinct DNA repair pathways depending on how they are rendered dysfunctional, and that ALT-NHEJ is a major pathway for processing of dysfunctional telomeres (Rai et al., 2010).

2.2.5 NHEJ-defective mouse models of cancers and leukemias

2.2.5.1 NHEJ in V(D)J recombination and CSR

In addition to DSBs generated by exogenous and endogenous DNA damaging agents, DSBs also occur as a consequence of specific physiological processes such as the V(D)J recombination in B and T-lymphocytes and the immunoglobulin CSR within immunoglobulin variable domains in B-lymphocytes during the development and maturation of the immune system (Ferguson and Alt, 2001, Revy et al., 2005). The organism recruits stringent DNA repair machinery to ensure the efficient repair of the damage or the elimination of the damaged cells. Failure to properly repair the DNA damage may cause chromosomal abnormalities, which in turn may lead to genomic instability and predispose the cells to malignant transformation.

The immune system provides a unique platform for understanding the NHEJ pathway because of its requirement for V(D)J recombination and CSR for development and maturation. In these systems, DNA damage is initiated by recombination activating gene 1 and 2 (RAG1/RAG2) in the case of V(D)J recombination, activation-induced cytidine deaminase (AID) in the case of CSR (Oettinger et al., 1990, McBlane et al., 1995, Petersen et al., 2001, Manis et al., 2002), that is uniquely expressed in specialized B- or T-lymphocytes. The rejoining of the broken DNA ends is then completed by C- and/or ALT-NHEJ pathway (Bassing et al., 2002). Notably, V(D)J recombination specifically recruits the C-NHEJ pathway components (Corneo et al., 2007). In contrast, approximately 50% of CSR events are completed by the ALT-NHEJ pathways (Soulas-Sprauel et al., 2007b, Yan et al., 2007, Han and Yu, 2008). Animal models and human conditions have demonstrated that defects in any of the C-NHEJ pathway components may cause immunodeficiency. The resultant erroneous DNA repair may predispose the cells to genomic instability and the development of cancer.

2.2.5.2 Defective C-NHEJ in immunodeficiency

Spontaneous mutant and genetically engineered animal models deficient for the various C-NHEJ components have in common impaired V(D)J recombination and consequent immunodeficiency, together with increased sensitivity to ionizing radiation.

Severe combined immune deficiency (SCID) mouse is a naturally occurring mutant mouse strain (Bosma and Carroll, 1991) which harbors a non-sense mutation in their highly conserved C-terminal part of DNA PKcs gene (Blunt et al., 1996, Araki et al., 1997). These mice lack mature B and T lymphocytes (Bosma and Carroll, 1991), accompanied by an increased cellular radiosensitivity (RS-SCIDs), indicative of a defect in DNA repair. Similarly, DNA PKcs knockout mice do not show overt cellular growth defects but exhibit immunodeficiency and ionizing radiation hypersensitivity (Gao et al., 1998, Taccioli et al., 1998, Kurimasa et al., 1999). Artemis-deficient mice resemble DNA PKcs-deficient mice, including a leaky SCID and increased cellular ionizing radiation sensitivity, supporting the idea that Artemis cooperates with DNA PKcs in a subset of C-NHEJ functions (Rooney et al., 2002).

Like the DNA-PKcs mutant SCID mice, Ku70 and Ku86 knockout mice demonstrate “leaky” immunodeficiency and are hypersensitive to irradiation (Nussenzweig et al., 1996, Zhu et al., 1996, Gu et al., 1997, Ouyang et al., 1997). In addition, they also show signs of growth retardation and extensive apoptosis of the newly generated neurons. Mice lacking either XRCC4 or DNA ligase IV die *in utero* with massive neuronal apoptosis and a complete block in lymphocyte development, suggesting the requirement for Ku, XRCC4 and DNA ligase IV in growth control and neuron development (Barnes et al., 1998, Frank et al., 1998, Gao et al., 1998). Mice lacking XLF are also immunodeficient and hypersensitive to ionizing radiation. However, they have modestly reduced lymphocyte numbers, nearly normal V(D)J recombination and moderately defective immunoglobulin heavy chain CSR (Li et al., 2008). Combined deficiency of ATM and XLF severely impairs V(D)J recombination and nearly blocks mouse lymphocyte development, indicative of the compensatory roles of ATM and XLF in C-NHEJ pathway (Zha et al., 2011), as discussed earlier in this chapter.

2.2.5.3 Involvement of ALT-NHEJ pathways in leukemia/lymphoma in mouse models

In the absence of C-NHEJ, the microhomology-based ALT-NHEJ is thought to be employed to ligate the broken DNA ends generated during V(D)J recombination and CSR.

Mice defective for one or more C-NHEJ components show various degrees of genomic instability. The absence of Ku, XRCC4, DNA ligase IV, XLF, Artemis, or DNA PKcs leads to the accumulation of DNA breaks and translocations in ES cells, fibroblasts or stimulated B cells (Guidos et al., 1996, Nacht et al., 1996, Karanjawala et al., 1999, Difilippantonio et al., 2000, Ferguson and Alt, 2001, Zhu et al., 2002, Rooney et al., 2003, Yan et al., 2007, Franco et al., 2008, Li et al., 2008, Boboila et al., 2010a). In the presence of the p53-null background, deletion of any one of the key components of the C-NHEJ pathway invariably leads to the early onset of very aggressive tumors, mostly pro-B-cell lymphomas, which generally harbor chromosomal translocations. Mice defective for *P53* and *Ku86* develop pro-B-cell lymphoma at an early age (Difilippantonio et al., 2000). These tumors display a specific set of chromosomal translocations and gene amplifications involving the immunoglobulin heavy chain IgH/Myc locus, reminiscent of Burkitt lymphoma. Combined deficiency in p53/XRCC4 or p53/DNA ligase IV results in live births. However, the offspring are immunodeficient and develop pro-B cell lymphomas (Frank et al., 2000, Gao et al., 2000). Mice lacking both Artemis and p53 develop pro–B cell lymphomas harboring N-myc-IgH, but not the Myc-Igh translocations observed in tumors in other C-NHEJ/p53 deficient mice (Rooney et al., 2004). XLF/p53-double-deficient mice are not markedly prone to pro-B lymphomas. However, like other C-NHEJ/p53-deficient mice, they still develop medulloblastomas (Li et al., 2008).

Recent studies based on C-NHEJ deficient mutant models also revealed that Ku or DNA ligase IV/XRCC4 are not required for, but rather suppress chromosomal translocations (Corneo et al., 2007, Soulas-Sprauel et al., 2007a, Yan et al., 2007, Boboila et al., 2010a). It has recently been reported that translocation breakpoint junctions are similar in wild-type and Ku or XRCC4 deficient mutants, including an unchanged bias toward microhomology. Complex insertions at some breakpoint junctions show that joining can be iterative, encompassing successive processing steps before joining, implying that ALT-NHEJ contributes to the translocation formation in mammalian cells (Simsek and Jasin, 2010).

Altogether, the development of leukemia/lymphoma in C-NHEJ deficient mouse models suggests that ALT-NHEJ pathways play important roles in the DSB repair in V(D)J recombination and CSR. Its low fidelity predisposes the cells to genomic instability and the development of malignancy. Further investigations of the molecular mechanisms underlying these pathways will provide insights into the roles of ALT-NHEJ in the occurrence of genomic instability and the development of cancer.

2.2.6 ALT-NHEJ in human cancer and leukemia

Many of the studies characterizing ALT-NHEJ have been conducted in a background of experimentally induced deficiency of components of the C-NHEJ pathway. This has drawn criticism that the results demonstrating ALT-NHEJ are biased by artificial experimental conditions that do not exist in reality. Many human cancers are characterized by recurrent chromosome abnormalities and microhomologous sequences have been identified at the breakpoint junctions of these abnormalities. Therefore, human cancers may represent model systems in which to study ALT-NHEJ.

2.2.6.1 Leukemia and lymphoma cells as models for the study of ALT-NHEJ

Like the SCID phenotype observed in mice, defective V(D)J recombination in humans causes arrest of B and T lymphocyte maturation, conferring severe combined immune deficiency (T-B-SCID). 70% of T-B-SCID patients have mutations in RAG1 or RAG2, which disables the

initiation steps in V(D)J recombination. The remaining 30% patients also show hypersensitivity to ionizing radiation, and therefore referred to as RS-SCID. They are caused by defects in the C-NHEJ pathway (de Villartay et al., 2003). So far, most genetic defects reported are found in the Artemis gene. In other cases, mutations in DNA ligase IV, XLF and DNA PKcs are reported. EBV-associated B-cell lymphomas and leukemia have been reported in patients with Artemis and DNA ligase IV mutations, respectively, indicative of the genomic instability associated with the impaired C-NHEJ (Riballo et al., 1999, Moshous et al., 2003). Patients with mutations in the gene encoding XLF also have greater chromosomal instability (Dai et al., 2003, Buck et al., 2006). These findings suggest that RS-SCID patients defective for C-NHEJ have elevated genomic instability which may predispose the cells to cancer. It is also likely that ALT-NHEJ among other error-prone pathways may drive genomic instability in these cases.

2.2.6.2 Microhomologies at breakpoints junction of recurrent alterations in cancer and leukemia

In vivo and *in vitro* assays in cancer and leukemia cells demonstrate increased errors following repair, with the majority of errors resulting from large DNA deletions occurring at the repair sites characterized by sequence microhomologies. Using *in vitro* end-joining assays based on repair of pUC18 plasmids containing a DSB in cell lines derived from myeloid leukemias, Gaymes et al. demonstrated a significant increase in errors characterized by increased size of deletions and microhomologies at the repair junctions further suggestive of the importance of ALT-NHEJ repair in these malignancies (Gaymes et al., 2002). Analyzing actual genomic deletions in tumors, Canning and Dryja found genomic deletions involving the retinoblastoma gene in 12 of 49 tumors from patients with retinoblastoma or osteosarcoma. They mapped the deletion breakpoints and sequenced 200 base pairs surrounding each deletion breakpoint in DNA from 4 tumor samples. Three deletions had termini characterized by direct repeats ranging in size from 4 to 7 base pairs (Canning and Dryja, 1989).

Recurrent chromosome translocations characterize leukemia and lymphoma and are specifically associated with their classification and prognosis. They occur frequently in both *de novo* and therapy-related in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Cloning of the genomic breakpoints in the common chromosome translocations in leukemia reveal that most of the genomic breakpoints tend to cluster in a restricted intronic region (Zhang and Rowley, 2006). In addition, sequencing of the translocation junctions identified regions of microhomology, strongly indicative of the involvement of ALT-NHEJ in the repair of DSBs and the generation of these chromosomal abnormalities (Reichel et al., 1998, Gillert et al., 1999, Strissel et al., 2000, Rassool, 2003, Zhang and Rowley, 2006; Wiemels and Greaves, 1999, Xiao et al., 2001, Reiter et al., 2003, Zhang and Rowley, 2006). An important example of such a study is sequencing of the TEL AML1 gene fusions found in pediatric leukemias and approximately 25% of adult acute B cell lymphomas. Analysis of the DNA sequence and structure surrounding the breakpoints revealed clues to their possible formation (Wiemels and Greaves, 1999). A long-distance inverse PCR strategy was used to amplify *TEL-AML1* genomic fusion sequences from diagnostic DNA from nine patients. Breakpoints were scattered within the 14 kb of intronic DNA between exons 5 and 6 of *TEL* and in two putative cluster regions within intron 1 of *AML1*. DNA sequences containing the breakpoint junctions exhibited characteristic signs of C- and ALT-NHEJ, including microhomologies at the breakpoints, small deletions and

duplications. Wiemels and Greaves concluded that the data was compatible with the possibility that *TEL-AML1* translocations occur by nonhomologous recombination involving imprecise, constitutive repair processes following DSBs (Wiemels and Greaves, 1999, Zelent et al., 2004).

2.2.6.3 Origins of DNA damage?

Genes such as *AML1*, *TEL* or *mixed lineage leukemia (MLL*) have been found rearranged with different partner genes in lymphoid and myeloid leukemias (Zelent et al., 2004). These translocations have been shown to correlate with sites of double-strand DNA cleavage by agents to which the cells or patients have been exposed, including exogenous rare-cutting endonucleases, radiomimetic compounds, and topoisomerase inhibitors (Greaves and Wiemels, 2003). The nature of the DNA damage leading to *MLL* translocations in leukemia in infants have been examined by several investigators (Greaves and Wiemels, 2003). The finding of identical *MLL* rearrangements in the leukemias from pairs of monozygotic twins where both twins were affected, but not in their constitutional DNA, established that *MLL* translocations in infant leukemias are non-hereditary, non-constitutional, *in utero* events. Furthermore, the most likely explanation was that cells with the translocation were transferred from one twin to the other *via* the placenta (Felix et al., 2000). The retrospective finding of leukemia-associated *MLL* genomic breakpoint junction sequences by PCR analysis of genomic DNAs contained in bloodspots on neonatal Guthrie cards of infants who were diagnosed later with leukemia showed that *MLL* translocations also occur *in utero* in the non-twin cases (Gale et al., 1997). Molecular cloning and analysis of *MLL* genomic breakpoint junctions sequences in infant leukemias suggested staggered and/or multiple sites of breakage as elements of damage and DNA repair by C- and/or ALT-NHEJ. This provided further evidence that DNA damage and repair underlie the formation of the translocations (Greaves and Wiemels, 2003, Gilliland et al., 2004). Because *MLL* translocations are much less frequent in *de novo* leukemias of older patients but frequent in leukemias following chemotherapeutic DNA topoisomerase II poisons, e.g., etoposide, it has been proposed that leukemia in infants may have an etiology resembling treatment-related cases (Gilliland et al., 2004). The chemotherapy-leukemia association in the treatment-related cases suggests that chromosomal breakage resulting from DNA topoisomerase II cleavage and attempted repair of DSBs may play a role in the formation of these translocations. The precision of the breakpoint junction sequences and the results of DNA topoisomerase II *in vitro* cleavage assays in treatment-related leukemias are consistent with the processing of 4-base, staggered DSB (Gilliland et al., 2004). In the infant leukemias, the breakpoint junction sequences and *in vitro* cleavage assays suggest a mechanism in which DNA topoisomerase II introduce separate single-stranded nicks in duplex DNA that are staggered by up to several hundred bases. This leads to a DNA damage-repair model in which various naturally occurring DNA topoisomerase II poisons induce DNA topoisomerase II-mediated damage in leukemia *in utero* (Gilliland et al., 2004). The large deleted regions observed in other infant cases are consistent with multiple sites of breakage or, alternatively, more extensive processing (Raffini et al., 2002).

2.2.6.4 Increased ALT-NHEJ activity in leukemia

In addition to increased DNA damage providing a substrate for error-prone repair and genomic instability, increased repair activity may also drive the acquisition of genomic alterations. Recently, Sallmyr at al. demonstrated increased activity of the ALT-NHEJ

pathway in chronic myeloid leukemia (CML) cells characterized by the oncogenic fusion tyrosine kinase, BCR-ABL (Gaymes et al., 2002, Sallmyr et al., 2008b). They showed that key proteins in the major C-NHEJ pathway, Artemis and DNA ligase IV, were down-regulated, whereas DNA ligase IIIα, and the protein deleted in Werner syndrome, WRN, are up-regulated in CML cells. Furthermore, they showed that DNA ligase IIIα and WRN form a complex that is recruited to DSBs, and that "knockdown" of either DNA ligase IIIα or WRN leads to increased accumulation of unrepaired DSBs, demonstrating that these DNA repair proteins contribute to their repair. To determine whether knockdown of either DNA ligase IIIα or WRN leads to differences in repair using DNA sequence microhomologies, Sallmyr et al. sequenced the breakpoint junctions of 15 repaired plasmids from each of the LacZα reactivation experiments. The majority (80%) of plasmids in CML cell line, K562 were repaired using DNA microhomologies of 1 to 6 bp. In contrast, plasmids from cells with reduced levels of either DNA ligase IIIα or WRN had a reduction in the overall percentage of microhomologies and these constituted 1 to 3 bp in length (DNA ligase IIIα, 25%; WRN, 40%) at the breakpoint junctions in repaired plasmids. Notably, in cell lines established from normal lymphocytes, end-joining assays reveal that the DSBs are repaired mainly using the C-NHEJ pathway. Furthermore, sequencing of the rare DSBs that were misrepaired (1 in approximately 10,000) revealed deletions of only a few base pairs. These results indicate that while ALT-NHEJ is possibly operative at very low levels in normal cells, altered DSB repair in CML cells may be caused at least in part by the increased activity of ALT-NHEJ repair pathway, involving DNA ligase IIIα and WRN. In AML characterized by expression of the constitutively activated receptor tyrosine kinase Fms Like tyrosine 3/Internal tandem duplication (FLT3/ITD), Sallmyr et al. reported that this constitutively activated tyrosine kinase initiates a cycle of genomic instability that is likely to promote both aggressive disease and resistance to therapy (Sallmyr et al., 2008a). Specifically, Sallmyr et al. showed that expression of FLT3/ITD induces increased reactive oxygen species production and that cells transformed by FLT3/ITD, including primary AML cells and cell lines established from FLT3/ITD-positive AML patients, have increased endogenous DSBs (Sallmyr et al., 2008a). Furthermore, repair of DSBs by NHEJ is less efficient and more error-prone in FLT3/ITD-expressing cells (Sallmyr et al., 2008a). More recently, Fan et al. reported that the steady state levels of Ku86 and to a lesser extent, Ku70, are significantly reduced in FLT3/ITD-expressing cells (Fan et al., 2010). In turn, there is a concomitant increase in the steady state levels of ALT-NHEJ components, PARP-1 and DNA ligase IIIα (Fan et al., 2010). Similar alterations in Ku86 and PARP-1 are also observed in FLT3/ITD knock-in mice, but increased levels of DNA ligase IIIα are only seen in the homozygote mice (Fan et al, 2010). Similar changes in C-NHEJ and ALT-NHEJ components are observed at the transcript level (Li et al., 2011) (**Figure 3**). In the FLT3/ITD mouse model, the impairment of C-NHEJ decreases the ability of cells to complete post-cleavage DSB ligation, resulting in failure to complete V(D)J recombination inhibiting B-lymphocyte maturation (Li et al, 2011). As a consequence of these changes in NHEJ proteins, the frequency of DNA sequence microhomologies and the size of deletions at repair sites are increased, reflecting the increased contribution of ALT-NHEJ to DSB repair. This suggests that FLT3/ITD signaling is involved in regulating both C- and ALT-NHEJ, directly or indirectly (**Figure 3**). Importantly, they reported that reducing the levels of DNA ligase IIIα in AML cells not only reduces the frequency of DNA sequence microhomologies and the size of deletions at repair sites but also increases the steady state levels of unrepaired DSBs, indicating that the ALT-NHEJ pathway is particularly important for the survival of FLT3/ITD expressing AML cells (Li et al., 2011).

2.2.7 ALT-NHEJ as therapeutic targets

Several lines of evidence suggest that both hereditary and sporadic cancers have abnormal level of DNA damage and repair responses that lead to the generation of structural chromosomal abnormalities and genomic instability, which are critical for survival, disease progression and resistance. The identification of defects in the DNA damage response between normal and cancer cells at the molecular level will guide the development of more targeted therapies by identifying biomarkers that are indicative of the abnormal DNA repair in cancer cells. To exploit the differences in the DNA damage response between normal and cancer cells, it will be necessary to characterize the DNA repair abnormalities and develop agents that target the abnormal DNA repair pathways that are specific for cancer cells, thereby reducing survival of cancer but not normal cells. In addition to participating in base excision and SSB repair, PARP-1 and DNA ligase IIIα also appear to be involved in ALT-NHEJ. In recent studies, we and others have shown that PARP-1 and DNA ligase IIIα are upregulated in certain cancers and leukemias (Chen et al., 2008, Sallmyr et al., 2008b, Fan et al., 2010, Li et al., 2011) (**Figure 4**).

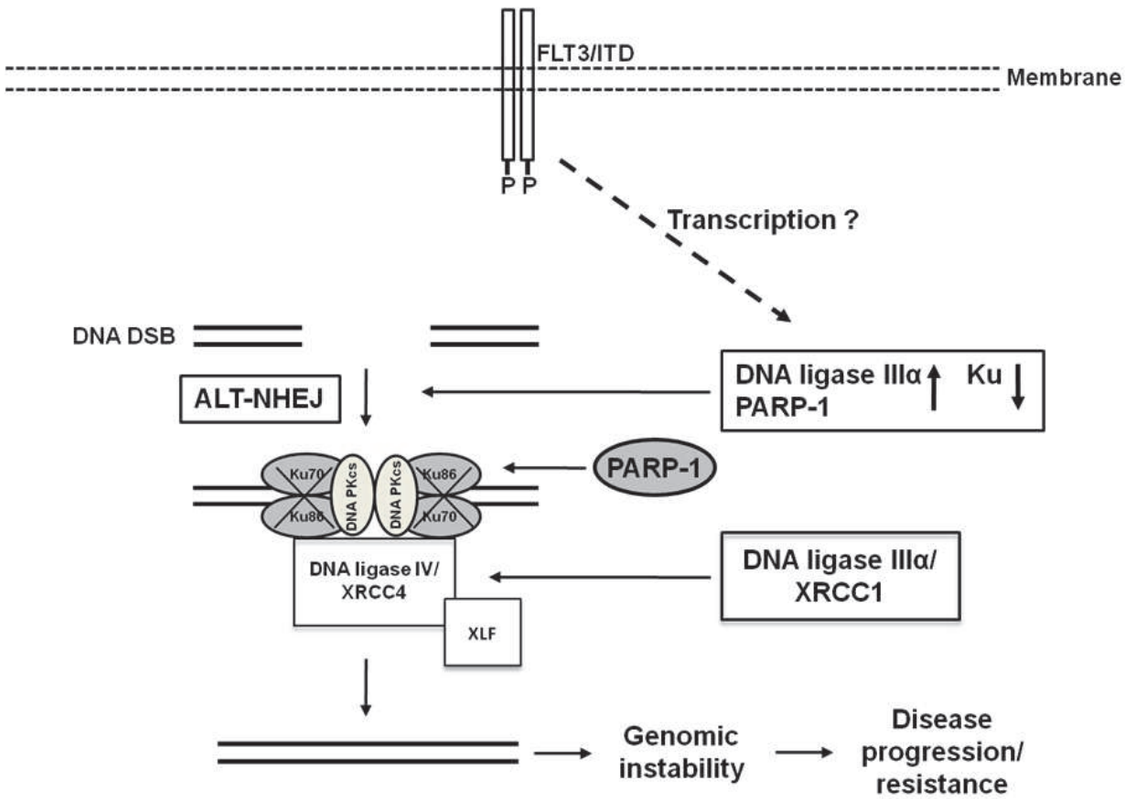

Fig. 3. Schematic for the mechanism of regulation of C- and ALT-NHEJ by FLT3/ITD. FLT3/ITD signalling leads to upregulation of DNA ligase IIIα and PARP-1 and downregulation of Ku70/86. The consequent increase in ALT-NHEJ activity promotes the acquisition of genomic changes that lead to disease progression or resistance to treatment.

2.2.7.1 PARP inhibitors

The abundant nuclear protein PARP-1 binds avidly to SSBs, an event that activates PARP-1 polymerase (Ame et al., 2004). Activated PARP-1 utilizes nicotinamide to synthesize

poly(ADP-ribose) polymers on itself and other nuclear proteins. Poly(ADP-ribosylated) PARP-1 serves as a recruitment factor for DNA ligase IIIα/XRCC1 and other factors involved in the repair of SSBs (Okano et al., 2003). Although there are other PARP family members, PARP-1 is the predominant enzyme that synthesizes poly(ADP-ribose) in response to DNA damage (Menissier de Murcia et al., 2003). The replication of DNA containing SSBs cause DSBs and so preventing the repair of SSBs by inhibiting PARP-1 results in an increase in DSBs. Since these replication-associated DSBs would normally be repaired by HR, cells that are defective in HR are hypersensitive to PARP inhibitors. Based on this rationale, potent and specific inhibitors of PARP were developed as therapeutic agents for inherited forms of breast and ovarian cancer as the PARP inhibitors should be cytotoxic for *BRCA* mutant tumors but not normal tissues with a functional *BRCA* allele (Bryant et al., 2005, Farmer et al., 2005). As expected, PARP inhibitors increased the cytotoxicity of a range of anti-cancer agents including temozolomide and ionizing radiation that cause SSBs (Tentori et al., 2002, Liu et al., 2008) and both *BRCA1*- and *BRCA2*-mutant cell lines were hypersensitive to PARP inhibitors in cell culture and mouse xenograft assays (Lord and Ashworth, 2008). These results formed the basis for a phase I clinical trial, which demonstrated that the PARP inhibitor AZD2281 exhibited antitumor activity in patients with ovarian and breast tumors resulting from either *BRCA1* or *BRCA2* mutations (Evers et al., 2008). The promising results from this clinical trial have prompted the evaluation of PARP inhibitors in combination with other cancer therapeutics in the treatment of different types of cancer.

Unfortunately, resistance to PARP-1 inhibitors has led to the failure of phase III clinical trials in triple negative breast cancers, and thus there is an urgency for elucidating the mechanisms by which resistance occurs in cells with defects in HR (Guha, 2011). One potential mechanism for resistance to PARP inhibitors in BRCA-deficient cells is that spontaneous or induced DSBs are rerouted for repair by error-prone mechanisms, including NHEJ, because the preferred mode of error-free repair by HR is unavailable (Venkitaraman, 2001). Patel et al. recently showed that in *BRCA2* deficient ovarian cancer cell lines PARP inhibitor treatment induces phosphorylation of DNA PK substrates and stimulates C-NHEJ selectively. Previous studies provided evidence for interplay between key C-NHEJ proteins and PARP-1: *(i)* PARP-1 can interact *in vitro* and *in vivo* with Ku (Galande and Kohwi-Shigematsu, 1999) and has been shown to compete with Ku80 for DNA ends *in vitro* (Wang et al., 2006), *(ii)* Ablation of C-NHEJ restores the survival of PARP-1-deficient cells treated with agents inducing DSBs (Hochegger et al., 2006). All together those results suggest that C-NHEJ and perhaps ALT-NHEJ could be involved in the genomic instability observed in HR-deficient cells treated with PARP inhibitors (Patel et al., 2011). Patel et al. showed that inhibiting DNA PK activity reverses the genomic instability induced by PARP inhibition in *BRCA2* deficient cells. Moreover, disabling C-NHEJ by using genetic or pharmacologic approaches diminished the toxicity of PARP inhibition in HR-deficient cells. These results not only implicate PARP-1 catalytic activity in the regulation of C-NHEJ and perhaps ALT-NHEJ in HR-deficient cells, but also indicate that deregulated C-NHEJ and perhaps ALT-NHEJ plays a major role in generating cytotoxicity and genomic instability in HR-deficient cells treated with PARP inhibitors (Patel et al., 2011). Recently Chen et al. showed that C-NHEJ protein DNA ligase IV was down regulated in cell lines derived from sporadic breast cancer (Chen et al., 2008). Thus, it would be important to evaluate C-NHEJ and ALT-NHEJ activity in *BRCA* deficient tumors in assessment of clinical response and resistance to PARP inhibitors.

2.2.7.2 DNA ligase inhibitors

DNA joining events are required for the completion of almost all DNA repair pathways. Thus, inhibitors of DNA ligase are predicted to sensitize cells to a variety of DNA damaging agents depending upon the inhibitor specificity for the three mammalian DNA ligases. Using computer-aided drug design based on the structure of human DNA ligase I in complex with nicked DNA, a series of small molecule inhibitors of human DNA ligases have been identified (Chen et al., 2008, Zhong et al., 2008). Briefly, an in silico data base of about 1.5 million commercially available small molecules was screened for candidates that were predicted to bind to a DNA binding pocket within the DNA binding domain (DBD) of human DNA ligase I. This binding pocket makes key contacts with nicked DNA (Chen et al., 2008). Out of 233 candidate molecules, 192 were assayed for their ability to inhibit human DNA ligase I but not T4 DNA ligase and for their ability to inhibit cell proliferation because human DNA ligase I is the major replicative DNA ligase. The *in vitro* DNA joining assays identified 10 small molecules that specifically inhibit human DNA ligase I by more than 50% at 100 mM, with 5 of these molecules also inhibiting the proliferation of cultured human cells. Since the amino acid sequences of the DBDs of human DNA ligases III and IV are closely related to that of the human DNA ligase I DBD, Chen et al. enquired whether the inhibitors of human DNA ligase I are also active against the other human DNA ligases. Molecules that inhibit DNA ligase I alone (L82), DNA ligase I and III (L67), and all three human DNA ligases (L189) *in vitro*, that were also active in cell culture assays, were further characterized. In accord with the screening strategy, all the ligase inhibitors with the exception of L82 act as competitive inhibitors with respect to nicked DNA. The structure of L67 and the other inhibitors consists of heterocyclic rings separated by a flexible linker. Interestingly, L67 and L189 are cytotoxic, whereas L82 is cytostatic. It is possible that this reflects the different mechanisms of inhibition. Alternatively, while inhibition of DNA ligase I alone is not toxic, inhibition of either DNA ligase III or DNA ligase IV alone or in combination with human DNA ligase I may be cytotoxic. Another interesting feature of the ligase inhibitors is that sub-toxic concentrations specifically potentiate the cytotoxicity of DNA-damaging agents in cancer cells **(Figure 4)**. As in cell lines expressing BCR-ABL and FLT3/ITD (Sallmyr et al., 2008b, Fan et al., 2010), DNA ligase IIIα is also overexpressed in cancer cell lines, whereas the levels of DNA ligase IV are reduced compared to a non-cancerous breast epithelial cell line (Chen et al., 2008). Together these results suggests that ligase inhibitors will not only provide a novel approach to delineating the cellular functions of these enzyme, but may also serve as lead compounds for the development of therapeutic agents that target DNA replication and/or repair (Chen et al., 2008).

Notably, these compounds exhibit different specificities for the three human DNA ligases *in vitro* and a subset of these molecules preferentially sensitize cancer cells to DNA alkylating agents and ionizing radiation, suggesting that they may have utility as lead compounds for the development of novel therapeutic agents. Notably, the ligase inhibitors would constitute an extremely versatile group of agents in that, depending on their specificity, they can be used to target a variety of DNA repair pathways that would be chosen based on the DNA damaging agent. For example, a DNA ligase IV specific inhibitor would sensitize cells with a functional C-NHEJ pathway to ionizing radiation whereas a DNA ligase III specific inhibitor would sensitize cancer cells that are dependent upon ALT-NHEJ to ionizing radiation and other agents that cause DSBs, such as PARP inhibitors (**Figure 4**).

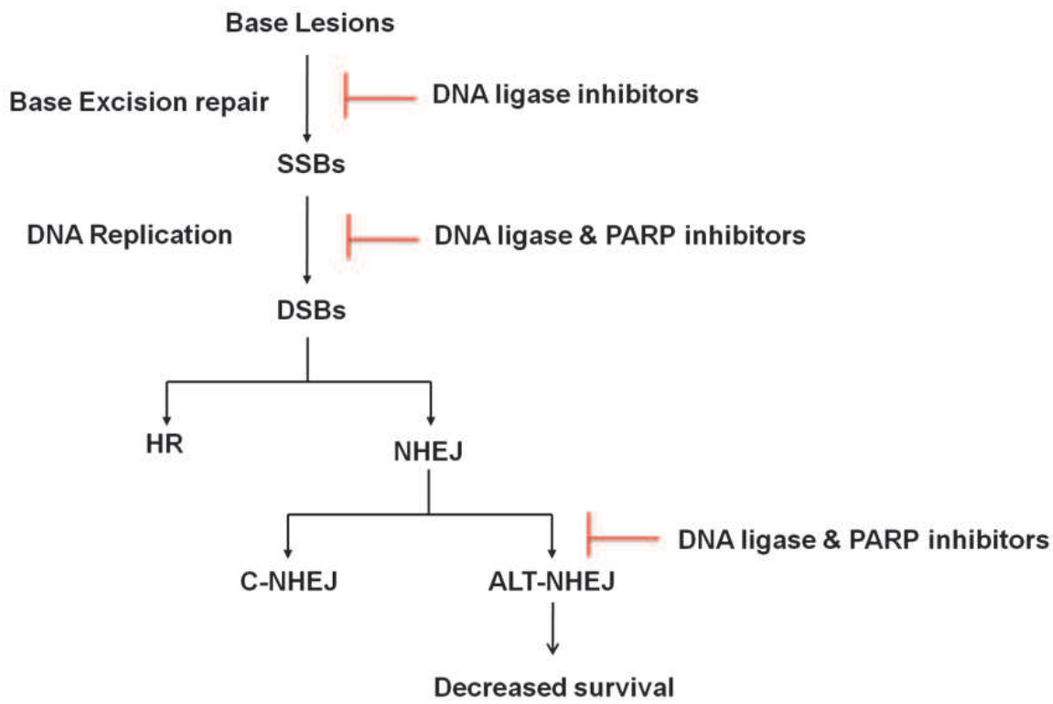

Fig. 4. Schematic of the effect of DNA ligase and PARP inhibitors on base excision repair, DNA replication and ALT-NHEJ in cancer and leukemia cells.

3. Conclusion

ALT-NHEJ is involved in the development of a variety of cancers, including leukemias, and is likely to play a key role in the generation of chromosomal abnormalities, including translocations, that drive cancer progression. It appears that cancer cells are more dependent on ALT-NHEJ for the repair of DSBs and survival, compared with normal cells. Thus targeting this pathway may be an attractive therapeutic strategy. Elucidation of the pathways components, and how they are regulated, will further guide the design of these therapies. Finally, investigation of the molecular mechanisms underlying abnormal DNA damage and repair in cancers and leukemias, together with the development of new animal models, will better our understanding of the complex relations between DNA repair and neoplastic transformation, which will provide new targets for the treatment of cancer.

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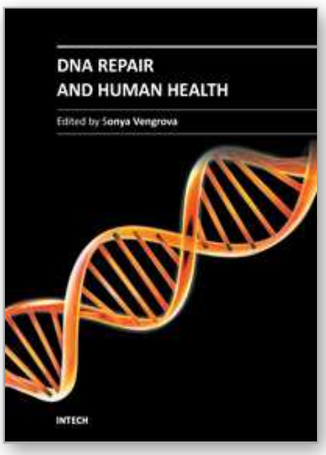

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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