

The endless tale of non-homologous end-joining

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DNA double-strand breaks (DSBs) are introduced in cells by ionizing radiation and reactive oxygen species. In addition, they are commonly generated during V(D)J recombination, an essential aspect of the developing immune system. Failure to effectively repair these DSBs can result in chromosome breakage, cell death, onset of cancer, and defects in the immune system of higher vertebrates. Fortunately, all mammalian cells possess two enzymatic pathways that mediate the repair of DSBs: homologous recombination and non-homologous end-joining (NHEJ). The NHEJ process utilizes enzymes that capture both ends of the broken DNA molecule, bring them together in a synaptic DNA-protein complex, and finally repair the DNA break. In this review, all the known enzymes that play a role in the NHEJ process are discussed and a working model for the co-operation of these enzymes during DSB repair is presented.

Keywords: DNA-PK, Ku70/80, XRCC4, Ligase IV, Artemis, XLF, Cernunnos, DSB, NHEJ, ATM, non-homologous end-joining, DNA double-strand break, V(D)J recombination

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The cell's response to DNA double-strand breaks

DNA double-strand breaks (DSBs) can be introduced by any agent that is capable of severing the DNA backbone. Such agents include endogenous factors like metabolic products and reactive oxygen species, and also exogenous factors like ionizing radiation. The induction of DSBs by ionizing radiation is of particular importance, since cells are continuously exposed to background radiation. The integrity of the DNA backbone is therefore under constant attack from both the inside and the outside of the cell. When both strands of the DNA backbone are severed at closely opposed positions, the DNA molecule will literally break apart. This leads to chromosome breakage, which, in turn, presents a major challenge for cell division. During division, chromosome fragments can be distributed unequally over daughter cells or they can be translocated to places in the genome where they do not belong. In many cases, cells that are the victim of such an attack suffer death. But in the worst-case scenario, deletion or translocation of chromosome fragments leads to inactivation of tumour suppressor genes or activation of oncogenes. Both events can trigger the onset of carcinogenesis in surviving cells [1, 2].

In view of the serious consequences that an unrepaired DSB can have in dividing cells, the cell needs to take rapid counter measures to (1) stop or delay cell division and (2) repair the DSB. The division of a cell is under the control of cell-cycle checkpoints in the G1, S, G2, and M phases, the activation of which leads to arrest of the normal division process [1]. It is generally assumed that cell-cycle arrest is necessary to provide the cell with enough time to adequately repair the DNA lesion. In the case of DSBs, cell-cycle arrest is thought to be primarily mediated by members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, which are activated upon the onset of DNA breakage [3-5]. In human cells, the activation of at least two PIKK signalling kinases – the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) proteins – leads to phosphorylation and activation of the central cell-cycle regulators p53, Chk1 and Chk2, which in turn facilitate G1 and G2 arrest via Cdk2/Cyclin E and Cdk2/Cyclin B1, respectively [4].

While the division of the DSB-containing cell is thus halted, several sets of enzymes work on the repair of the DSB. Two distinctly different pathways that mediate DSB repair have been identified in mammalian cells: homologous recombination (HR) and non-homologous end-joining (NHEJ) [1, 6] (Figure 1). The HR pathway is a very accurate repair mechanism, in which a homologous stretch of DNA on a sister chromatid serves as a template to guide repair of the broken strand. NHEJ, on the other hand, is a

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potentially less accurate form of DSB repair, in which the two termini of the broken DNA molecule are processed to form compatible ends that are directly ligated. Under certain circumstances, this latter method can result in the loss of nucleotides and hence in the loss of genomic integrity. At this point it is not completely clear which factors determine the use of HR versus NHEJ, but the cell-cycle stage must play an important role in this decision. After all, the homologous template necessary for HR is only present during the S and G2 phases of the cell cycle. It is therefore believed that NHEJ, although potentially inaccurate, is the prevailing repair pathway during G1 and M phases.

Non-homologous end-joining: the basic mechanism

The molecular mechanism of the NHEJ process looks deceptively simple: a set of enzymes mediates (1) the capture of both ends of the broken DNA molecule, (2) the formation of a molecular bridge that brings the two DNA ends back together; and (3) the subsequent re-ligation of the broken DNA molecule. In reality, the NHEJ process is rather complex and requires a tightly coordinated interplay between the key enzymes to timely initiate each subset of the entire process. Many of these interactions have not been

fully investigated and are still poorly understood.

The NHEJ process (Figure 1B) is initiated by the binding of a protein complex, the Ku70/80 heterodimer, to both ends of the broken DNA molecule. It is currently believed that the association of a DNA end with the Ku heterodimer creates a scaffold for the assembly of the other NHEJ key enzymes. In an early stage of NHEJ-mediated DSB repair, the DNA-Ku scaffold attracts the DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}) to the DSB. This kinase has multiple roles, including the formation of a synaptic complex which brings both DNA ends together. Once the two DNA ends have been captured and tethered in a protein complex consisting of Ku and DNA-PK_{CS}, non-ligatable DNA termini must be processed before final repair of the DSB can take place. Several enzymes have been identified, including nucleases and polymerases, that are able to either remove or fill-in single-stranded, non-compatible overhangs. It is this step in the NHEJ process that is considered to be responsible for the occasional loss of nucleotides associated with NHEJ-mediated repair. Finally, the ligase IV/XRCC4 complex catalyses ligation of the processed DNA ends. This ligation reaction may be enhanced by the presence of the recently discovered XLF/Cernunnos protein.

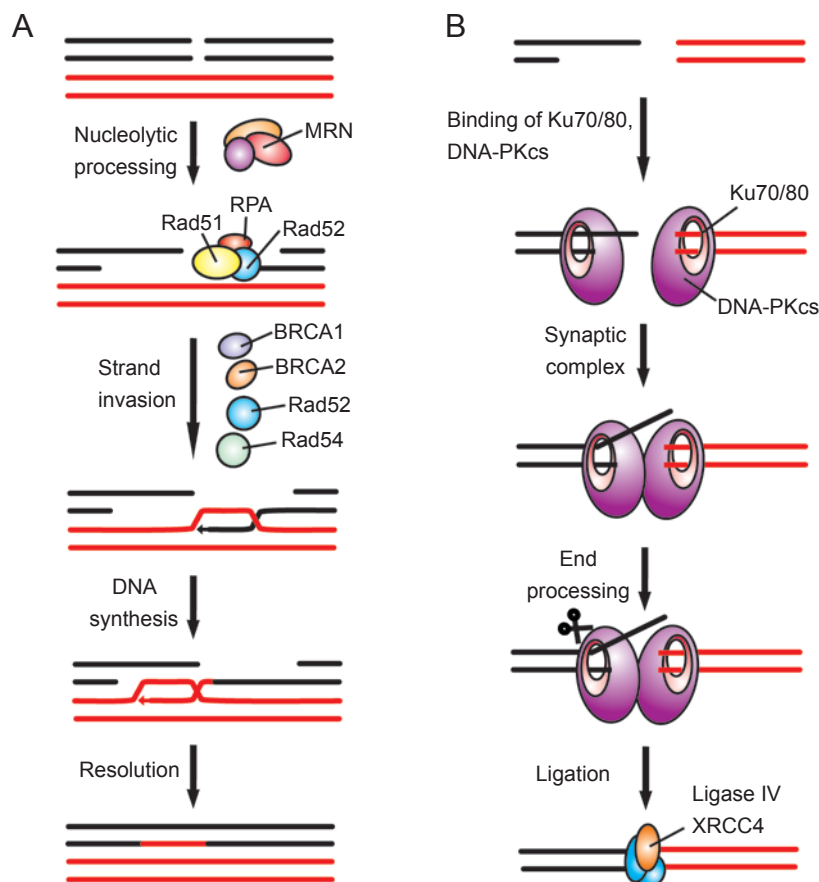


Figure 1 Simplified overview of homologous recombination (HR) and non-homologous end-joining (NHEJ). **(A)** HR utilizes a homologous stretch on a sister chromatid to accurately repair the DSB. DNA ends are first processed in order to create single-strand overhangs, a process that is likely mediated by the MRN (Mre11/Rad50/Nbs1) complex. Rad51, Rad52, and RPA associate with these overhangs, followed by the formation of a joint molecule by the damaged and undamaged strands. Template guided DNA synthesis and resolution of the two strands then complete repair of the DSB. **(B)** NHEJ brings the ends of the broken DNA molecule together by the formation of a synaptic complex, consisting of two DNA ends, two Ku70/80 and two DNA-PK_{CS} molecules. Non-compatible DNA ends are processed to form ligatable termini, followed by repair of the break by the ligase IV/XRCC4 complex.

Non-homologous end-joining and V(D)J recombination: where immunology and DNA repair meet

DSBs are not only introduced by exposure to mutagenic agents or radiation. They are also continuously generated in developing B- and T-lymphocytes. This is due to the fact that the genes which encode immunoglobulins (Ig) or T-cell receptors (Tcr) are not present in an active form in these lymphocytes. The formation of active Ig or Tcr genes requires the combination of gene segments by a process called V(D)J recombination, which mediates the excision, relocation, and re-ligation of these gene segments in a manner that utilizes several of the NHEJ key enzymes [6, 7]. The sheer endless number of combinations that can be made with those segments accounts for the great potential of the immune system to recognize a wide range of immunogens (Figure 2).

The gene segments are classified into different groups, which are called variable (V), diversity (D), and joining (J) segments. Active IgH genes are made by the selection and coupling of a D and a J segment, followed by the coupling of the DJ assembly with a V segment. This coupling takes place by the introduction of a DSB at the edge of the selected gene segment, removal of the intervening DNA, and ligation of the gene segment with its designated partner. DSBs are introduced during V(D)J recombination by the RAG1 and RAG2 (Recombination-Activating Genes) proteins at specific sites: the recombination signal sequences. The ends of the gene segments that are to be joined in order to form the mature Ig or Tcr gene are called coding ends. Coding ends are characterized by a hairpin structure that needs to be opened before end-joining can proceed (Figure 2).

The opening of the coding end hairpin structures is mediated by the Artemis nuclease, which is essential for V(D)J recombination [8, 9]. The Artemis protein forms a complex with the NHEJ factor DNA-PK_{CS}. This association activates the Artemis endonuclease and enables opening of the coding end hairpins, facilitating subsequent ligation of the V, D, or J gene segments [10]. Ligation of these segments requires the presence of the NHEJ core factors ligase IV and XRCC4, indicating that repair of the RAG-induced DSBs during V(D)J recombination is mediated by the NHEJ process [6, 7].

The early stages of non-homologous end-joining

It is generally assumed that the Ku70/80 heterodimer and the DNA-PK_{CS} kinase are recruited to the DSB site in the earliest stages of the NHEJ process. Several recent studies have demonstrated that Ku70/80 and DNA-PK_{CS} migrate towards artificially introduced DSBs in living cells in a

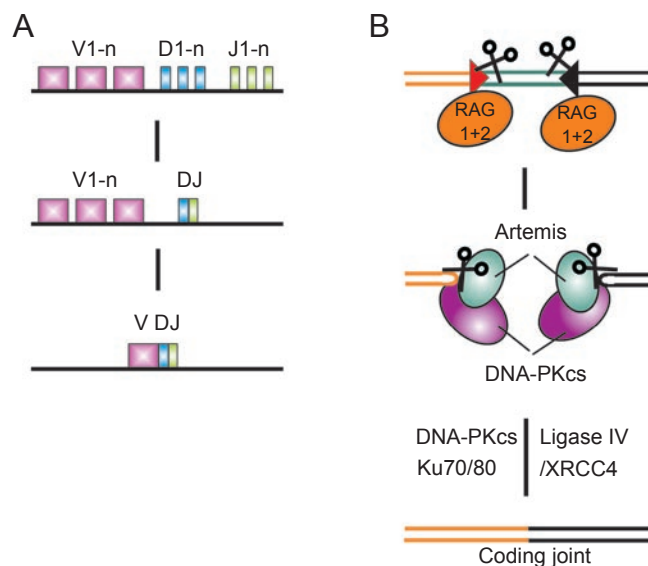


Figure 2 Simplified overview of V(D)J recombination. **(A)** Genes that encode immunoglobulins or T-cell receptors are not present in an active form in developing B- and T-lymphocytes, but need to be formed by the combination of gene segments. This process is called V(D)J recombination. Gene segments are classified into three groups: variable (V), diversity (D), and joining (J) segments. In the case of an IgH gene, D and J segments are first joined, followed by the combination of the DJ assembly with a V segment. **(B)** Gene segments are joined by the introduction of a DSB at the edges of selected segments by the RAG 1 and RAG 2 proteins, followed by removal of the intervening DNA and ligation of the segments. Before ligation can take place, the typical hairpin structure of the coding ends needs to be opened by the endonuclease Artemis. V(D)J recombination requires the NHEJ core enzymes (DNA-PK_{CS}, Ku70/80, ligase IV, and XRCC4), indicating that ligation of the gene segments is mediated by the NHEJ process.

manner of seconds after the onset of the damage [11, 12].

The extraordinary high affinity of Ku70/80 for DNA termini and its abundant concentration in the cell nucleus make it very likely that this protein dimer associates with the ends of a broken DNA molecule immediately after the introduction of the break. Crystallography studies of Ku70/80 revealed that the heterodimer has an open ring-shaped structure, composed of one Ku70 (73 kDa) and one Ku80 (86 kDa) protein which both constitute part of the ring [13]. This hetero-dimerization is mediated mainly by the central regions of both the Ku70 and Ku80 proteins [13, 14] (Figure 3A). The opening of the ring accommodates a DNA helix and this feature allows the Ku70/80 dimer to slide over the ends of a broken DNA molecule, thus accounting for the high affinity of Ku70/80 for DNA termini [13]. The central regions of both the Ku70 and the Ku80 protein, which are located on the inside of the ring, assume a three-dimensional structure that sterically fits the DNA helix,

but makes little direct contact with the DNA backbone or bases [13] (Figure 3A). Indeed, careful examination of the dynamics of Ku80 molecules at DSB sites in living cells has revealed that Ku does not bind rigidly to DNA ends, but that there is a continuous dynamic exchange between DNA-bound Ku and free, unbound Ku [12].

The Ku-DNA complex acts as a scaffold for the association of a 460-kDa serine/threonine kinase: DNA-PK_{CS}. This interaction between Ku and DNA-PK_{CS} is thought to be mediated by the Ku80 carboxy-terminus [15-17] (Figure 3A). The three dimensional structure of the DNA

- Ku - DNA-PK_{CS} complex has not been resolved by crystallography studies, but electron microscopy images are available at a 25 Å level [18-20]. Interestingly, these images show dimeric structures in which two DNA ends are tethered by a protein complex consisting of two Ku70/80 dimers and two DNA-PK_{CS} molecules, suggesting that DNA-PK_{CS} is capable of forming a ‘bridge’ between two DNA molecules. This hypothesis is further evidenced by atomic force microscopy studies, which demonstrate the presence of DNA-PK_{CS} at juxtaposed DNA ends [21]. In addition, biochemical experiments have shown that DNA

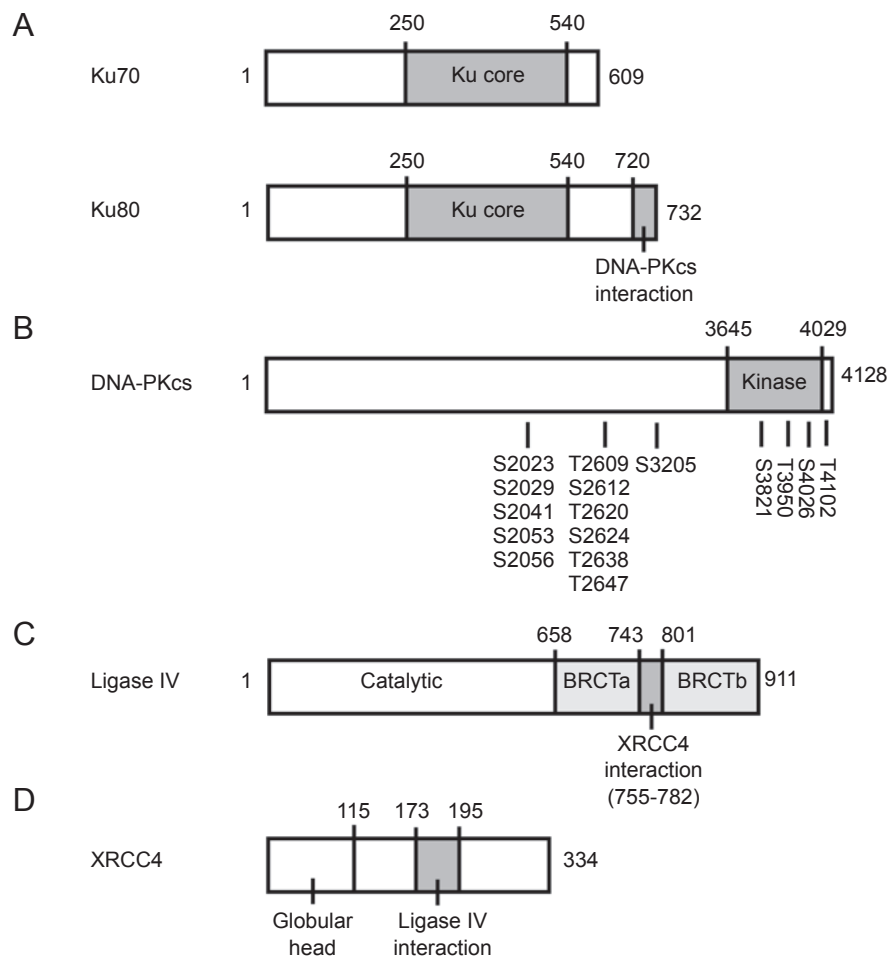


Figure 3 Schematic representations of the human NHEJ core enzymes. Drawings are not to scale. **(A)** The central regions of the Ku70 and the Ku80 protein (the ‘Ku core’ domains, roughly encompassing residues 250-540) mediate both the hetero-dimerization of Ku70 and Ku80 and the interaction of the Ku70/80 heterodimer with the DNA helix [13]. The association of the Ku70/80 heterodimer with DNA-PK_{CS} is thought to be mediated by the extreme carboxy-terminus of Ku80 [16, 17]. **(B)** The serine/threonine kinase domain of DNA-PK_{CS} is located in the carboxy-terminus, between residues 3645 and 4029. All known phosphorylation sites are indicated: the 2056 cluster, the 2609 cluster, and the carboxy-terminal cluster [35]. **(C)** The ligase IV protein possesses a BRCT tandem that encompasses residues 658-911, but the domain that mediates the interaction between ligase IV and XRCC4 is located in between the two BRCT regions, spanning residues 755-782 [58]. Several domains necessary for the catalytic activity of ligase IV are situated in the amino-terminal region [54]. **(D)** The first 115 amino acids of the XRCC4 protein form a globular head [59], while the rest of the molecule forms a long stalk which interacts with ligase IV between residues 173 and 195 [58].

molecules can be held together by the DNA-PK_{CS} protein [22]. Collectively, these data led to the current notion that DNA-PK_{CS} mediates tethering of the ends of a broken DNA molecule by binding to the DNA-Ku scaffold and by subsequently forming a synaptic complex that juxtaposes two DNA termini, two Ku dimers and two DNA-PK_{CS} molecules (Figure 1B).

The intimate association of DNA-PK_{CS} with both a DNA terminus and a Ku dimer is necessary for the activation of the DNA-PK_{CS} serine/threonine kinase, located at the carboxy-terminus of the DNA-PK_{CS} molecule (Figure 3B). The role of the DNA-PK_{CS} kinase within the NHEJ process is not completely resolved at this point, although it is clear that this activity is required for efficient NHEJ [23]. No evidence is available that the DNA-PK_{CS} kinase activity is necessary for the formation of the synaptic complex as described above. Several targets of the DNA-PK_{CS} kinase have been identified *in vitro*, including components of the NHEJ repair complex (XRCC4 and Ku70/80) and the cell-cycle regulator p53 [24, 25]. The fact that DNA-PK_{CS} is able to phosphorylate these targets suggests a role in either cell cycle arrest or regulation of the NHEJ process itself. However, the *in vivo* significance of DNA-PK_{CS} mediated phosphorylation of XRCC4, Ku70/80, and p53 has not been conclusively demonstrated or is disputed [26-28].

The most extensively studied target of the DNA-PK_{CS} kinase is DNA-PK_{CS} itself. Currently, there are 16 amino-acid residues identified within the DNA-PK_{CS} molecule that can be autophosphorylated by the DNA-PK_{CS} kinase (Figure 3B). It has been well established that this autophosphorylation activity is important for the efficient progression of the NHEJ process, since mutation of several autophosphorylation sites results in diminished DSB repair and sensitivity of cells to DSB-inducing ionizing radiation [29-34].

How does DNA-PK_{CS} autophosphorylation facilitate NHEJ-mediated DSB repair? The phosphorylation status of the DNA-PK_{CS} molecule is known to influence its conformation and dynamics at the synaptic complex [35]. The presence of the large DNA-PK_{CS} molecule in its unphosphorylated form at the DNA termini effectively blocks the access of processing enzymes and ligases to the synaptic complex and inhibits effective ligation of the two DNA ends [36-38]. Obviously, this blockage needs to be relieved before the NHEJ process can progress further. It has been demonstrated that autophosphorylation of DNA-PK_{CS} is required to introduce a change in the DNA - DNA-PK_{CS} complex which relieves the blockage and allows processing and ligation of the DNA termini [37-39]. Recently, it has been shown that DNA-PK_{CS} autophosphorylation can occur in trans at the synaptic complex, suggesting that autophosphorylation takes place when the synapsis between the two DNA ends with bound Ku and DNA-PK_{CS} is established

[40]. Taken together, these findings gave rise to the current notion that the presence of unphosphorylated DNA-PK_{CS} protects the DNA termini against premature degradation or ligation until both ends of the broken DNA molecule are properly brought together in the synaptic complex. Trans autophosphorylation then introduces a conformational

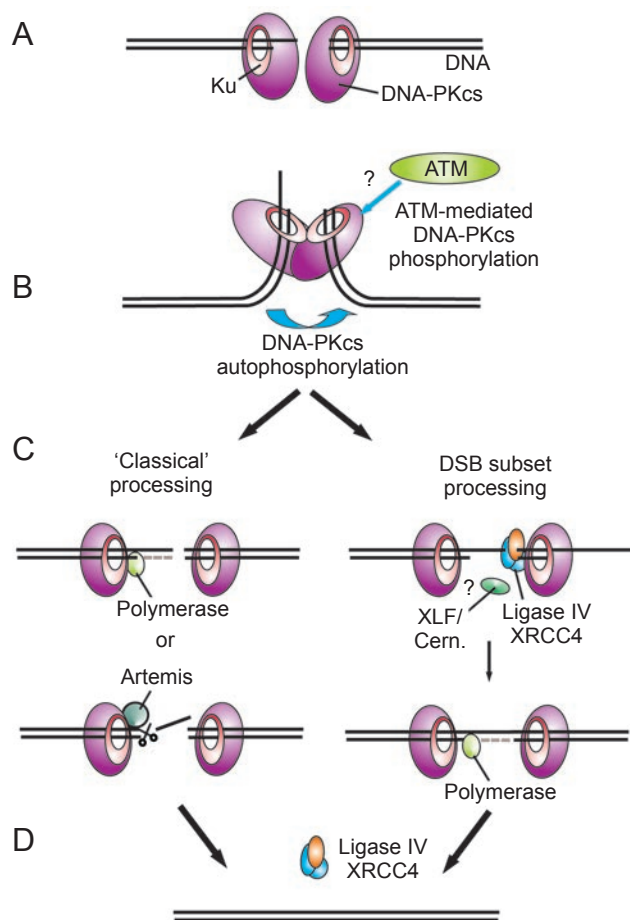


Figure 4 A model for NHEJ. **(A)** The Ku70/80 heterodimer associates with the two ends of the broken DNA molecule. This DNA-Ku scaffold attracts DNA-PK_{CS}, which protects the DNA termini against degradation and premature ligation. **(B)** The DNA-PK_{CS} molecules on both DNA ends form a synaptic complex which tethers the DNA ends. Trans DNA-PK_{CS} autophosphorylation then introduces a conformational change that makes the DNA termini accessible for other NHEJ enzymes. In addition to autophosphorylation, ATM-mediated DNA-PK_{CS} phosphorylation may play a role in this conformational change. **(C)** Non-compatible DNA termini need to be processed before ligation can proceed. This can be done in the 'classical' way, by either filling (polymerases) or resection (Artemis) of single-strand overhangs, followed by **(D)** ligation of the blunt ends by ligase IV/XRCC4. Alternatively, in a subset of DSBs with partially complementary overhangs, ligase IV/XRCC4 and XLF/Cernunos mediate the joining of one single-strand overhang with the opposite DNA end, followed by filling of the gap by polymerases.

change in the DNA-PK_{CS} molecule that results in accessibility of the DNA ends for processing enzymes and ligases (Figure 4). To date, the nature of this conformational change has not been further investigated. Analysis of the dynamics of phosphorylated and unphosphorylated DNA-PK_{CS} at DSB sites *in vivo* revealed that the exchange between DNA-bound and free DNA-PK_{CS} progresses at a much lower rate in the case of unphosphorylated DNA-PK_{CS}, suggesting that the autophosphorylation-induced conformational change increases the rate of dissociation and re-association of DNA-PK_{CS} at DNA termini. This increased exchange rate is likely to also increase the window of time that is available for processing or ligating enzymes to associate with the DNA ends [11].

Future research will have to unravel the role that each DNA-PK_{CS} phosphorylation site plays during the conformational changes that facilitate access to the DNA ends. It appears that the phosphorylation sites in the DNA-PK_{CS} molecule are arranged in three major clusters: five residues in the 2 056 cluster, six residues in the 2 609 cluster, and four residues in the carboxy-terminus (Figure 3B). The 2 609 cluster is the most extensively studied. Inhibition of phosphorylation of the residues in this cluster severely decreases DNA end processing [30]. This effect is strongest when phosphorylation of all residues is inhibited, suggesting that the residues within the 2 609 cluster cooperate in facilitating DNA end accessibility. Only one study has been devoted to the role of the 2 056 cluster, which demonstrates that phosphorylation of this cluster may inhibit end processing [32]. It therefore appears that the 2 056 and 2 609 clusters have opposing activities, although this finding will need further experimental evidence. Of the carboxy-terminal cluster, only the 3 950 residue has been subjected to close examination. This phosphorylation site is located within the serine/threonine kinase domain of DNA-PK_{CS} (Figure 3B) and mutation of the 3 950 residue with phosphomimic aspartic acid results in abrogated V(D)J recombination, sensitivity to ionizing radiation, and hence in reduced DSB repair [33]. In view of its location within the kinase domain, it is tempting to speculate that the 3 950 residue may modulate DNA-PK_{CS} autophosphorylation by regulating kinase activity.

Recent reports have shown that the 2 609 and 2 647 residues of DNA-PK_{CS} are still being phosphorylated in the absence of a functional DNA-PK_{CS} kinase after the introduction of DSBs *in vivo* [11, 41]. These findings suggest that some DNA-PK_{CS} phosphorylation sites can be phosphorylated by kinases other than the DNA-PK_{CS} kinase. One study finds the availability of the PIKK kinase ATM necessary for phosphorylation of the 2 609 and 2 647 residues [41]. Another study finds no absolute requirement for ATM, but does conclude that DNA-PK_{CS}

phosphorylation occurs to a limited extent under conditions that exclude autophosphorylation [40]. Since ATM is known to be present at DSB sites *in vivo* [42], it is conceivable that the full array of phosphorylation events that lead to the conformational changes in the DNA-PK_{CS} molecule is mediated by both the ATM kinase and the DNA-PK_{CS} kinase [35] (Figure 4).

The later stages of non-homologous end-joining

It is not easy to make a clear distinction between the early and later stages of the NHEJ process, because it is possible that the enzymes that act in the later stages of NHEJ (processing enzymes and ligases) are already associated with the synaptic repair complex since the initiation of the process. The DNA-Ku scaffold is able to attract more enzymes besides DNA-PK_{CS} (as will be discussed later), suggesting that early association of processing enzymes, ligase IV/XRCC4 and XLF/Cernunnos with the synaptic complex is possible. However, relevant knowledge about the exact timing of the recruitment of these enzymes to the repair complex is lacking at this point. Because ligation cannot proceed before proper processing of the DNA ends is completed, and because processing cannot proceed without phosphorylation of DNA-PK_{CS}, we arbitrarily denote processing and ligation as the ‘later’ stages of NHEJ-mediated repair.

Reactive oxygen species and ionizing radiation introduce a rather heterogeneous array of DSBs. The simplest DSB form is one that consists of two blunt DNA termini. Such termini would not require processing and could be directly ligated. But in the majority of cases the two DNA termini will not be directly compatible because at least one of the strands will have a 3′ or 5′ single-strand overhang. The size of this overhang depends on the relative distance between the positions where each of the two strands of the DNA backbone was severed. There are two ways by which a single-strand DNA overhang can be removed: either by using the nucleotide sequence of the overhang as a template to synthesize the complementary strand or by resection of the overhang at the position where the single-strand segment extends from the double-strand helix (Figure 4).

Several enzymes capable of synthesizing a complementary strand by adding nucleotides have been shown to play a role in NHEJ. Human terminal deoxynucleotidyltransferase (TdT) co-localizes with Ku at DSB sites, indicating that this enzyme may be attracted by the DNA-Ku scaffold and functions as a processing factor during NHEJ mediated DSB repair [43, 44]. DNA polymerase μ is also suggested to interact with Ku and stimulates end-joining *in vitro* [45]. Similarly, depletion of DNA polymerase λ from a HeLa extract reduced end-joining in an *in vitro* assay [46]. Notably,

all these enzymes have functions during other cellular processes and cannot be considered specialized NHEJ factors. It appears that the NHEJ process utilizes DNA modifying factors that belong to the general enzymatic pool of the cell. At present, it is not known which factor or process determines which modifying enzyme is to be used during the processing of a specific set of DNA termini.

As far as resection of single-strand overhangs is concerned, only one enzyme has been demonstrated to perform this task during NHEJ: the endonuclease Artemis. This enzyme was initially recognized as an essential factor during V(D)J recombination, facilitating the opening of hairpin structures at coding ends [8-10]. However, since Artemis deficiency not only causes impairment of V(D)J recombination but also sensitivity to ionizing radiation, it is likely that Artemis plays an important role during repair of radiation-induced DSBs. The Artemis protein by itself only displays a 5'-3' exonuclease activity. In order to acquire the endonuclease activity that mediates hairpin opening during V(D)J recombination, Artemis must associate with the DNA-PK_{CS} molecule [10]. This association of Artemis and DNA-PK_{CS} is most likely responsible for the recruitment of Artemis to the synaptic repair complex, although it is not known during which phase of the NHEJ process Artemis is drawn to the DSB site. Both the DNA-PK_{CS}-independent exonuclease activity and the DNA-PK_{CS}-dependent endonuclease activity of Artemis can be relevant for processing of DNA termini during repair of radiation-induced DSBs. It is therefore unclear whether the association of Artemis with DNA-PK_{CS} is only necessary for the physical attachment of Artemis to the repair complex or that it is also essential to expand the nucleolytic potential of the Artemis enzyme.

After exposure of cells to ionizing radiation, Artemis is (hyper)phosphorylated by both the ATM and DNA-PK_{CS} kinases [47]. At present it is not yet resolved whether this phosphorylation is required for activity of the Artemis nuclease, because contradicting information on this topic has been presented. One report shows the involvement of DNA-PK_{CS} in phosphorylation of the carboxy-terminal part of Artemis, thereby relieving auto-inhibition of the Artemis endonuclease activity [48]. Contrary, another report suggests that the DNA-PK_{CS} kinase is not essential for Artemis endonuclease activation, but rather for DNA-PK_{CS} auto-phosphorylation which facilitates access of Artemis to the DNA termini as explained in the previous section [49].

Processing of DNA ends is not limited to filling or resection of single-stranded overhangs. It can also include the addition of 5' phosphate groups which are necessary for the ligation reaction. This activity is mediated by the mammalian polynucleotide kinase (PNK) in an XRCC4- and DNA-PK_{CS}-dependent manner [50]. In certain cases, 3' phosphoglycolates must be removed before ligation can take

place. This task can be performed by the apurinic/apurinic endonuclease APE1, the tyrosyl-DNA phosphodiesterase TDP1, the polynucleotide kinase PNK and the endonuclease Artemis [51, 52]. Although these four enzymes have very different catalytic activities, together they are able to recognize a wide variety of DNA substrates [51].

After proper processing of the DNA termini, the stage is set for the final repair of the broken DNA molecule. During the previously discussed steps of the NHEJ process, the DNA ends have been tethered in a synaptic complex and non-compatible overhangs were transformed into 5' phosphorylated, ligatable ends (Figure 4). Ligation of these processed DNA termini is mediated by a complex of two proteins: ligase IV and XRCC4. The importance of these enzymes during DSB repair is clearly demonstrated by the fact that deficiency in either of them leads to embryonic lethality in mice and that ligase IV- and XRCC4-deficient cells are sensitive to ionizing radiation [6].

The catalytic domain of the 100 kDa, ATP utilizing ligase IV protein is located in the amino-terminal region, which shares distinct homology with the other main mammalian ligases: ligase I and III [53, 54]. Contrary to the amino terminus, the carboxy terminus of ligase IV is a rather unique structure and contains two regions that harbour a BRCT (BRCA1 carboxy-terminus like) motif: an approximately 95 amino-acid spanning sequence that is known to be involved in protein-protein interaction, especially with BRCT domains in other proteins [55] (Figure 3C). Interestingly, however, the interaction between ligase IV and XRCC4 is not thought to be mediated by the BRCT motifs themselves, but by the sequence that lays in between the two BRCT domains, encompassing amino-acids 755 to 782 [56-58].

The crystal structure of XRCC4 reveals that this protein has a globular amino-terminal head and a long carboxy-terminal stalk [59]. The amino-terminal head most likely interacts with DNA and the carboxy-terminal region between amino acids 173 and 195 mediates the interaction between XRCC4 and ligase IV [58] (Figure 3D). The three-dimensional structure of the ligase IV/XRCC4 complex shows two XRCC4 molecules that are positioned side by side with the two carboxy-terminal stalks extending more or less in a parallel fashion, while one ligase IV molecule is associated with the combined stalks of the XRCC4 dimer [58, 60]. In view of this structure, it is likely that the amino-terminal heads of the XRCC4 molecules mediate an interaction with the DNA helix, while the ligase IV enzyme repairs the DSB.

Several studies have demonstrated interactions between the ligase IV/XRCC4 complex and the Ku70/80 heterodimer, suggesting that the ligase IV/XRCC4 complex is attracted to the synaptic repair complex by the DNA-Ku

scaffold in a manner similar to the recruitment of DNA-PK_{CS} and several of the processing enzymes [12, 61-64]. The BRCT motifs of ligase IV may be involved in the interaction of the ligase IV/XRCC4 complex with Ku70/80 [64]. One *in vitro* study has shown that the ligation activity of ligase IV/XRCC4 increases 20-fold upon interaction with Ku70/80, suggesting that Ku not only recruits but also activates ligase IV/XRCC4 [61]. It is unknown at present, during which stage of the NHEJ process ligase IV/XRCC4 associates with the DNA-Ku scaffold. It is possible that the ligase IV/XRCC4 complex binds simultaneously with DNA-PK_{CS} to the DNA termini and awaits its turn until DNA-PK_{CS} phosphorylation has mediated accessibility of the DNA ends. On the other hand, ligase IV/XRCC4 may join the repair complex only after tethering and processing of the DNA termini.

Several authors have introduced interesting models in which the ligase IV/XRCC4 complex not only facilitates the finishing touch of the NHEJ process but also has an important function in the initiation of DNA end processing in a subset of DSBs. In these models, Ku and ligase IV/XRCC4 cooperate in the positioning of incompatible, unprocessed DNA ends in such a way that the overhanging strand of one DNA terminus is joined with the opposite DNA terminus (Figure 4). This creates a structure in which two duplex DNA strands are joined by a short single-stranded stretch, which can be filled in by polymerases [51, 65-69]. This kind of alternative processing may be possible when the single-strand overhang is partially complementary with the opposite DNA end. Clearly, in such cases, end processing and ligation cannot be considered to be two distinctly separated processes but they are in fact joined in one cooperative effort.

In 2006, two research groups simultaneously identified a novel factor with a clear significance for NHEJ. One group named this factor Cernunnos [70], and the other group named it XRCC4-like factor (XLF) [71]. As the latter name suggests, this 33 kDa protein displays sequence and structure homology with XRCC4. The XLF/Cernunnos factor was discovered after reports of a cell line that exhibited DSB repair and V(D)J recombination deficiencies (2BN), but could not be complemented with the known NHEJ genes [72]. XLF/Cernunnos was found to rescue the defects of the 2BN cells, demonstrating the involvement of XLF/Cernunnos in NHEJ-mediated DSB repair [71, 73]. Information on the factors that influence the recruitment of XLF/Cernunnos to the synaptic repair complex is limited, although it has been demonstrated that this recruitment may depend on the availability of ligase IV/XRCC4 [71] and Ku70/80 (Ken-ichi Yano, *EMBO Rep* in progress).

The XLF/Cernunnos factor interacts with the ligase IV/XRCC4 complex, suggesting that XLF/Cernunnos

stimulates or modulates the ligation efficiency of ligase IV/XRCC4 [71]. Recent studies demonstrate that XLF/Cernunnos stimulates the ligation of non-compatible DNA ends [66, 74]. In the presence of XLF/Cernunnos, two DNA substrates (one with a blunt end and one with a 3' overhanging end) were ligated at the overhanging strand, leaving the other strand unligated [66]. The non-ligated strand could theoretically be a substrate for a polymerase that fills in the gap and facilitates complete repair of the break. By stimulating ligation of non-compatible ends XLF/Cernunnos may be able to prevent resection of single-strand overhangs, thereby greatly contributing to the preservation of the DNA sequence and the prevention of nucleotide loss during NHEJ.

Concluding remarks

The functions of the NHEJ core factors and the processing enzymes cannot be understood without a thorough knowledge of the multiple interactions that these factors have with each other. The DNA - Ku scaffold seems to play an especially important role in the NHEJ process, since it is known to associate with all the other factors: DNA-PK_{CS}, ligase IV/XRCC4, XLF/Cernunnos, and the processing polymerases. The NHEJ pathway can be roughly divided into two phases: an early phase and a late phase, although it may well be possible that contributors of the early phase may be present and active during the late phase and vice versa. The early phase constitutes the recruitment of Ku70/80 to the ends of the broken DNA molecule, the binding of DNA-PK_{CS} to the DNA - Ku complex, the formation of a synaptic complex that tethers the two DNA ends, and the regulation of DNA end accessibility by phosphorylation events within the DNA-PK_{CS} molecule (Figure 4). After the DNA termini are made accessible by DNA-PK_{CS} phosphorylation, the stage is set for the late phase: processing and ligation of the DNA termini. Classical models depict the processing step as a process that is separate from and precedes the activity of the ligase IV/XRCC4 complex. Modern insights, however, have revealed that at least for a certain subset of DSBs, especially those with at least partially complementary overhangs, ligase IV/XRCC4 and Ku may align the DNA ends prior to processing. This mode of action, perhaps stimulated by XLF/Cernunnos, would allow ligation of one strand, leaving a gap at the other strand that can be filled in by a polymerase (Figure 4). It will be one of the important tasks for future researchers to investigate the plausibility of this combined processing/ligation model.

The working model for the NHEJ mechanism is far from resolved and many questions remain to be answered. We provide here a brief overview of some issues that will need to be addressed in the near future: (1) The fate of the

Ku70/80 molecule after repair of the DSB. To our knowledge, no experiments have been published that shed light on the fate of the Ku70/80 ring after recruitment of the NHEJ key enzymes. The high affinity of Ku70/80 for DNA termini makes it unlikely that it would dissociate from the DNA helix, unless some conformational change is introduced in the Ku70/80 molecule via its interaction with one of the other NHEJ enzymes. It is possible that the Ku70/80 ring still encircles the DNA helix after repair of the DSB. In that case, the heterodimer will have to be removed by the action of a still unidentified protease. (2) The nature of the conformational change that takes place after DNA-PK_{CS} autophosphorylation. Although it has been shown that phosphorylation of the 2 609 cluster is required for effective DSB repair, the effects brought on by phosphorylation of the other 10 residues within the DNA-PK_{CS} molecule remain somewhat elusive. Next to nothing is known about the influence of DNA-PK_{CS} phosphorylation on its three-dimensional structure. (3) The requirement for Artemis phosphorylation during activation of its endonuclease. The relevance of DNA-PK_{CS}-mediated phosphorylation of Artemis during DSB repair is a matter of current debate and the function of ATM-mediated phosphorylation of Artemis is even less well understood. These three examples are merely a selection of the current questions within the field and they clearly show that there are still many opportunities for scientists to fill knowledge gaps and add to the seemingly endless tale of non-homologous end-joining.

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