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DNA-PK: a dynamic enzyme in a versatile DSB repair pathway

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Abstract

DNA double stranded breaks (DSBs) are the most cytoxic DNA lesion as the inability to properly repair them can lead to genomic instability and tumorigenesis. The prominent DSB repair pathway in humans is non-homologous end-joining (NHEJ). In the simplest sense, NHEJ mediates the direct re-ligation of the broken DNA molecule. However, NHEJ is a complex and versatile process that can repair DSBs with a variety of damages and ends via the utilization of a significant number of proteins. In this review we will describe the important factors and mechanisms modulating NHEJ with emphasis given to the versatility of this repair process and the DNA-PK complex.

Keywords

DNA double strand breaks; non-homologous end-joining; Ku70/80; DNA-PKcs; XRCC4; DNA Ligase IV; XLF

1. Introduction

Protecting its genome is paramount for each organism because DNA alterations such as mutations and chromosomal aberrations can lead to disease, tumorigenesis, or cell death [1]. A cell encounters a large number of DNA lesions each day that jeopardize the integrity of the genome, with the DNA double strand break (DSB) being the most toxic. The deleterious nature of DSBs is underscored by the fact that a single unrepaired DSB can cause cell death and misrepaired DSBs can result in chromosomal aberrations such as translocations and deletions, which can result in a loss of heterozygozity leading to genomic instability and subsequent malignant transformation. DSBs can be induced by a variety of means including intrinsic sources such as byproducts of cell metabolism and oxidative damage and extrinsic sources like ionizing radiation and chemotherapeutic agents [2, 3]. Although typically deleterious by nature, developing lymphocytes intentionally and systematically induce DSBs during V(D)J recombination and class switch recombination for the development of T and B cells [4]. To counter DSBs, organisms have developed a complex response that includes recognition of the broken DNA molecule, cellular signaling including modulating the cell cycle, and ultimately repair of the DNA lesion [1, 5]. Two prominent pathways mediate the repair of DSBs in mammalian cells termed homologous recombination (HR) and non-

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homologous end-joining (NHEJ). HR mediates DSB repair by utilizing a homologous stretch of DNA to guide repair of the broken DNA strand. Since an easily accessible homologous template is found on a sister chromatid, HR is believed to be primarily active during the S and G2 phases of the cell cycle. As the name indicates, NHEJ mediates the direct re-ligation of the broken DNA molecule. Since NHEJ does not require a homologous template, it is not restricted to a particular phase of the cell cycle. It should be noted that homology independent repair in the absence of the canonical NHEJ factors also occurs. This alternative end-joining pathway (Alt-NHEJ), also named back-up NHEJ (B-NHEJ) and microhomology-mediated end-joining (MMEJ), is associated with DSB repair in which deletions occur at the repair junction and typically utilizes microhomologies distant from the DSB to mediate the repair [6]. In this review we will focus on the classical/canonical NHEJ pathway and hereafter “NHEJ” refers to this DSB repair pathway.

2. General mechanism of NHEJ

NHEJ is an amazingly versatile pathway that can select specific enzymes which bind to, process, and finally mediate the direct re-ligation of a wide range of DSBs including those that are complex, have incompatible ends, and contain base damages [7–10]. The general mechanism of NHEJ include the following steps: (I) Recognition of the DNA double strand break by the Ku70/80 heterodimer (Ku); (II) Assembly and stabilization of the NHEJ complex at the DNA damage site; (III) Bridging of the DNA ends and promotion of end stability; (IV) Activation of the DNA dependent protein kinase catalytic subunit (DNA-PKcs) kinase activity; (V) DNA end processing, if required; (VI) Ligation of the broken ends by the DNA Ligase IV-X-ray cross complementing protein 4 (XRCC4) complex with the assistance of the XRCC4-like factor (XLF, also named Cernunnos); and (VII) Dissolution of the NHEJ complex and completion of the repair process (Figure 1). It should be noted that although these steps are listed sequentially, the order in which a number of these steps occur in particular steps II–V are unknown. In this review, we will highlight the important NHEJ proteins and mechanisms modulating NHEJ with emphasis given to the DNA-PK complex and the versatility of this DSB repair pathway.

3. Ku70/80

NHEJ is initiated by the sensing and binding of the DSB by the Ku heterodimer, which consists of the Ku70 and Ku80 subunits [11, 12]. Ku is an abundant protein that has an extremely high affinity for dsDNA ends that forms a ring-shaped protein that slides onto the ends of the broken DNA molecule in a sequence independent manner [13–16]. Ku70/80 binds to the sugar backbone of DNA and not to the bases, which explains its ability to bind to DNA in a sequence independent manner [14]. Depending on the length of the DNA substrate, multiple Ku molecules can slide onto naked DNA in vitro [17, 18]. However, recent data shows that typically only one Ku heterodimer is bound to each end of the DSB in vivo [19]. Following induction a DSB, Ku70/80 binds to the DNA damage site within seconds of its creation and does so in all cell cycle phases [11, 19, 20]. Once bound to the DSB, Ku then performs its primary function in NHEJ, which is to serve as a scaffold to recruit the NHEJ machinery to the DNA lesion. The Ku heterodimer directly interacts with each canonical NHEJ factor, DNA-PKcs [12], XRCC4 [11, 21, 22], DNA Ligase IV [21]

and XLF [23], as well with the majority of the DNA end processing factors [7]. The recruitment of the NHEJ machinery to the site of the DSB does not occur in a step-wise sequential manner but via a dynamic assembly [24, 25]. For example, the NHEJ factors required for the terminal ligation step, XRCC4, DNA Ligase IV, and XLF, localize to DSBs independently of DNA-PKcs, which had long thought to be required for their recruitment to DSBs [11, 24]. The canonical NHEJ factors appear to collectively stabilize the entire NHEJ machinery at the DNA damage site and do so by a number of protein-protein interactions between themselves [25, 26].

A secondary function that the Ku heterodimer performs at DSBs is a general role in binding to and maintaining the stability of the ends of the broken DNA molecule in all cell cycle phases [20]. Ku maintains the two ends of the broken DNA molecule together via forming a synaptic complex in vitro and is also required for the positional stability of DSB ends in vivo [27–30]. The role of Ku in maintaining and stabilizing DSB ends is likely to protect them from non-specific processing. Specifically, Ku has been shown to block DNA end processing enzymes including exonuclease 1 and the Mre11/Rad50/Nbs1 complex in vitro [31]. Blocking non-specific processing of a DSB is of importance because it protects against chromosomal aberrations and genomic instability. This is supported by the fact that Ku deficient cells have severe chromosomal instability following DSB induction in S phase cells, suggesting that Ku has a general function in protecting the genome even when HR is the likely preferred DSB repair pathway [32]. Although Ku plays a role in maintaining DSB ends in all cell cycle phases, it should be noted that Ku-mediated end-joining may also be detrimental to cell survival, in particular end-joining of DSBs at replication forks. For example, Ku is required for the cell killing of cells following combined treatment with camptothecin and inhibition of the ataxia-telangiectasia mutated (ATM) protein [19], treatment of HR-deficient cells with Poly (ADP-ribose) polymerase (PARP) inhibitors [33], and in Fanconi Anemia (FA)-deficient cells [34].

4. DNA-PK

After sensing and binding to the DSB, Ku quickly recruits DNA-PKcs to the site of the DNA break. Similar to Ku70/80, recruitment of DNA-PKcs to DSBs occurs within seconds of their creation [12]. The interaction between Ku70/80 and DNA-PKcs requires the presence of dsDNA and the complex formed at the DSB consisting of DNA, Ku70/80, and DNA-PKcs is referred to as “DNA-PK” [35]. DNA-PKcs is a member of phosphatidylinositol-3 (PI-3) kinase-like kinase family (PIKK), which also includes the two DNA damage responsive proteins, ATM and ATM and Rad3- related protein (ATR) [36, 37]. The N-terminal region of DNA-PKcs is composed of HEAT (Huntington-elongation-A-subunit-TOR) repeats that likely serve as a protein-protein interaction interface and the C-terminal region of the protein contains the PI3 kinase domain, which is flanked N-terminally by the FAT (FRAP, ATM, TRRAP) domain and C-terminally by the FATC (FAT C-terminal) domain [38, 39]. Structural studies of DNA-PKcs show that the N-terminal portion of the protein produces a pincer-shaped structure that forms a central channel that likely binds to dsDNA and the C-terminal domains form a crown structure that sits on top of the pincer-shaped structure [40, 41]. Binding of DNA-PKcs to the DNA-Ku complex results in translocation of the Ku heterodimer inward on the dsDNA strand and ultimately results in

activation of the DNA-PKcs kinase activity [42, 43]. Once Ku recruits DNA-PKcs to the DSB ends, it has been shown that the large DNA-PKcs molecule also forms a distinct structure at the DNA termini that forms a synaptic complex responsible for holding the two ends of the broken DNA molecule together [27–29]. This synaptic complex consisting of DNA-PKcs and Ku is stable at DNA termini and blocks processing by nucleases and ligases and ultimately is required for DNA-PKcs kinase activation [29, 44]. It is likely that Ku70/80 recruits DNA-PKcs to the DSB via multiple contacts between the two proteins, which is supported by predictions from low resolution structure of the DNA-Ku70/80-DNA-PKcs complex [45, 46]. Small angle X-ray scattering analysis shows the Ku80 C-terminal region may play a role in retaining DNA-PKcs at DSB ends and keeping the DNA-PK complex in a synaptic complex at the DSB site [47]. Although the C-terminal region of Ku80 helps retain DNA-PKcs at DSB termini, it is not required for the ability of DNA-PKcs to localize to DSBs *in vivo* as previously believed [48–51]. The central cavity formed by the N-terminal region of DNA-PKcs results in DNA threading through the channel and ultimately stabilization of the DNA-PKcs-Ku-DNA complex and it is this portion of the protein that is required for the ability of DNA-PKcs to interact with the Ku-DNA complex [40, 41, 52].

5. DNA-PK kinase activity

As previously stated, DNA-PKcs recruitment to the DSB results in translocation of the Ku heterodimer inward on the dsDNA allowing DNA-PKcs to interact directly access DSB end, which results in activation of the catalytic activity of the enzyme [42, 43]., DNA-PKcs has no to limited kinase activity in the absence of Ku70/80 and DNA, thus making it truly a DNA-dependent protein kinase [53, 54]. The mechanism by which binding to the Ku-DNA complex stimulates the catalytic activity of DNA-PKcs is not clearly understood. It is likely that multiple regions/motifs of the protein play a role in this process. Low resolution structures showed that binding to the Ku-DNA complex induces a conformational change in the FAT and FATC domains surrounding the PIK3 kinase domain and this conformation change is predicted to result in the alteration of the catalytic groups and/or the ATP binding pocket of DNA-PKcs and ultimately full activation of its kinase activity [45, 46, 55]. Surprisingly, the N-terminus also plays a role in modulating the enzymatic activity of DNA-PKcs [52, 56]. Deletion of the N-terminal region of DNA-PKcs and N-terminally restraining DNA-PKcs results in spontaneous activation of its kinase activity suggesting that the N-terminus keeps DNA-PKcs basal activity low and that a perturbation of the N-terminus results in a conformational change that results in an increase in basal kinase activity.

The kinase activity of DNA-PKcs is essential for NHEJ, but the exact role of the enzymatic activity of DNA-PKcs plays in NHEJ is not fully understood [57]. In vitro, DNA-PKcs can phosphorylate each of the canonical NHEJ factors including Ku70/80 [58, 59], XRCC4 [60, 61], DNA Ligase IV [62], and XLF [63], but surprisingly none of these phosphorylations are required for NHEJ. DNA-PKcs has also been shown to phosphorylate a number of other factors implicated in NHEJ and the DNA damage response, including Artemis, polynucleotide kinase/phosphatase (PNKP), the histone H2AX and p53 [9, 64, 65]. But, considering these factors are also phosphorylated by ATM, the role of DNA-PKcs-mediated phosphorylation of these proteins is not exactly clear. However, it was recently found that phosphorylation of the implicated NHEJ factor Werner (WRN) by DNA-PKcs is required

for efficient DSB repair, possibly identifying a DNA-PKcs mediated phosphorylation of a substrate that is important for NHEJ [66]. Furthermore, a number of new in vivo substrates of DNA-PKcs have emerged [65]. Proteins that are phosphorylated by DNA-PKcs following DNA damage include Akt/PKB [67, 68], the nuclear receptor 4A [69], heat shock protein HSP90α [70, 71], and the scaffold attachment factor A [72], but it is not known if phosphorylation of any of these proteins is important for NHEJ or the cellular response to DSBs. In respect to the NHEJ pathway, the best characterized DNA-PKcs substrate is DNA-PKcs itself as it autophosphorylate a number of residues in different regions of the polypeptide [73–76]. Autophosphorylation of DNA-PKcs results in kinase inactivation and dissociation from the DNA-Ku70/80 complex in vitro, but even the specific role that DNA-PKcs autophosphorylation plays in NHEJ is not completely understood [73, 77].

6. Phosphorylation of DNA-PKcs

Following induction of a DSB, DNA-PKcs is phosphorylated on more than 40 sites including at a number of phosphorylation clusters (Discussed sites outlined in Figure 2) [78]. The best characterized DNA-PKcs phosphorylation cluster is the threonine 2609 (Thr2609) cluster. The Thr2609 cluster was original identified as an autophosphorylation site but further analyses revealed that the Thr2609 cluster is primarily targeted by ATM and ATR in response to DSBs and replication stress, respectively [79–82]. Phosphorylation of the Thr2609 cluster is important for NHEJ as ablating the phosphorylation of these sites via alanine substitutions results in severe radiosensitivity and diminished DNA end-joining ability in vitro [79, 81, 83, 84]. Furthermore, DNA-PKcs^{3A} knock-in mutant mice lacking a functional Thr2605 cluster (human Thr2609) confer early lethality and congenital bone marrow failure [85]. Cells derived from DNA-PKcs^{3A} mice are highly sensitive toward replication stress agents and impair in multiple repair pathways including HR and FA. These findings demonstrated the critical function and requirement of Thr2609 cluster phosphorylation in vivo under physiological conditions. Protein biochemistry and structure studies suggest that DNAPKcs phosphorylation at the Thr2609 cluster causes a large conformational change of DNA-PKcs, which promotes its dissociation from the Ku70/80 heterodimer and release from DSB ends [12, 47]. Additionally, Thr2609 cluster phosphorylation and/or conformational change of DNA-PKcs facilitate its association with other DNA repair molecules. For example, the coordination between ATM and Artemis in DSB repair requires DNA-PKcs phosphorylation at the Thr2609 cluster as phosphorylated DNAPKcs recruits Artemis to DNA ends and facilitates its endonuclease activity [86]. This notion is further supported by in vivo evidence that a functional Thr2609 cluster in conjunction with ATM kinase is able to promote the coding end joining during V(D)J recombination, a process requiring Artemis endonucleases activity [87].

The other well characterized phosphorylation cluster of DNA-PKcs is the serine 2056 (Ser2056) cluster [88, 89]. Serine 2056 is a bona fide autophosphorylation site in response to DSBs in vivo and phosphorylation of the Ser2056 cluster is important for NHEJ as ablating phosphorylation of this cluster causes increased radiosensitivity and less efficient DSB repair. The Ser2056 and Thr2609 phosphorylation clusters may play opposing roles in regard to protecting the ends of the DSB. Phosphorylation at Ser2056 limits DNA end processing whereas the Thr2609 promotes it [88, 90]. A number of phosphorylation sites

have been found in the N- and C-terminal regions of DNA-PKcs. Phosphorylation at threonine 946 and serine 1004 inhibits NHEJ but does not affect the enzymatic activity of DNA-PKcs [91]. Three phosphorylation sites have been shown to directly affect the kinase activity of DNA-PKcs. A phosphorylation site has also been identified in the kinase domain of DNA-PKcs at threonine 3950 [74]. Ablation of this site by alanine substitution does not affect the kinase activity of DNA-PKcs but the phospho-mimic aspartic acid substitution results in ablation of DNA-PKcs kinase activity, implicating that autophosphorylation at this site may be a method to directly turn off DNA-PKcs kinase activity. Phosphorylation of DNA-PKcs in its N-terminus at serines 56 and 72 also results in abrogation of DNA-PKcs enzymatic activity, but how phosphorylation of these two sites regulates the kinase activity of DNA-PKcs is unknown [91]. Finally, serine 3205 is phosphorylated in response to IR and this phosphorylation is mediated by ATM similar to the Thr2609 cluster [91]. Although much has been learned about how the phosphorylation status of DNA-PKcs regulates the functionality of the protein, the exact role of these phosphorylations in NHEJ and other DSB responses is still unclear and will continue to serves as a research topic of great interest.

7. Other factors regulating DNA-PKcs

Recent work in the field has also focused on factors that regulate NHEJ and NHEJ factors, in particular proteins that modulate DNA-PKcs. In this section we will highlight a number of factors which have been found to regulate DNA-PKcs. DNA-PKcs phosphorylation at the Thr2609 cluster has also been implicated in DNA-PKcs association with the epidermal growth factor receptor (EGFR). EGFR is one of the key determinants of the radioresponse in cancer radiotherapy as elevation of EGFR frequently correlates with radioresistance in cancer cells [92]. The underlying mechanism was revealed by the findings that EGFR and the downstream PI3K-Akt signaling pathway modulate DNA-PKcs activation and radiosensitivity [93, 94]. It was found that IR induces nuclear translocation of wild type EGFR but not mutants carrying oncogenic mutations at the tyrosine kinase domain, and that wild type EGFR binds to DNA-PKcs and promotes DSB repair [94]. A subsequent study further revealed that ATM-mediated DNA-PKcs Thr2609 phosphorylation is a critical prerequisite for EGFR function in radioresistance. Alanine substitution at Thr2609, but not at Ser2056, blocks the interaction between DNA-PKcs and EGFR and EGFR-mediated radioresistance [95]. Further, ATM deficiency or ATM kinase inhibition attenuates the EGFR dependent radioresponse.

Akt kinase signaling has also been implicated to regulate DNA-PKcs activation and DSB repair [68, 93, 96]. Specific findings include nuclear Akt activation (as monitored by Ser473 phosphorylation) promotes its direct interaction with DNA-PKcs through the C-terminal domain of Akt. As a result, nuclear Akt activation facilitates DSB accumulation of DNA-PKcs and DSB repair and Akt kinase inhibition radiosensitizes DNA-PKcs and Ku80 proficient cells [68]. In addition, it was found that IR-mediated Akt activation requires erbB receptors and DNA-PKcs, whereas EGF-mediated Akt activation is independent of erbB receptors and DNA-PKcs [96]. It is seemingly that there are both similarity and distinction of IR and EGF signaling in the modulation of EGFR and Akt activities for DSB repair. Further investigations are needed to clarify the coordination of EGFR and Akt kinases in DNA-PKcs dependent DSB repair. The knowledge generated will be beneficial for the

design and implementation of anti-EGFR, Akt, and DNA-PKcs strategies in cancer radiotherapy implication.

Several lines of recent evidence also indicate that DNA-PKcs activation is affected by chromatin status and modulators such as β isoform of heterochromatin protein 1 (HP1). HP1 is known to bind to histone H3 trimethyl Lys9 (H3K9me3) for constitutive heterochromatin [97, 98], which affects many DNA activities including transcription, replication, and DSB repair [99]. In attempt to identify additional protein lysine methylations, a proteomic screening using the chromodomain (known to bind H3K9me3) of HP1β as the bait was developed and DNA-PKcs was identified as one of interacting proteins of HP1β [100]. Mass spectrometry analysis identified that DNA-PKcs methylation occurred at three lysine residues (K1150, K2746, K3248) upon DNA damage and that methylation is necessary to mediate DNA-PKcs interaction with HP1β [100]. K-to-R (non-methyl) mutation of DNA-PKcs or depletion of HP1β impairs DSB repair and radioresistance, indicating that DNA-PKcs methylation and/or its association with HP1β affects DSB repair. Although the detailed mechanism remains to be clarified, DNA-PKcs methylation is likely required for HP1β recruitment to DSB sites and/or facilitates the DSB repair process. In agreement, it was reported that HP1β is phosphorylated by Casein Kinase II (CK2) at Thr51 upon DSBs, and that HP1β phosphorylation decreases its affinity to H3K9me at heterochromatin and promotes HP1β mobilization to DSB sites [101]. CK2 kinase is a versatile kinase involved in many cellular regulations including chromatin modulation in DNA damage sensing and repair [102, 103]. Two recent studies further suggested that CK2 is required for DNA-PKcs activation upon DSBs as CK2 depletion disrupts DNA-PK complex formation at DSBs, attenuates IR-induced DNA-PKcs activation, and facilitates IR-induced cell killing [104, 105]. Taken together, these results strongly suggest that CK2 and HP1β dependent chromatin remodeling plays a prominent role in DNA-PKcs activation and DSB repair. It is likely that CK2-dependent HP1β phosphorylation and DSB translocation induces chromatin relaxation for the full activation of DNA-PKcs, although the detailed kinetic and sequential order of these events remains to be clarified. It is interesting to note that there is a direct interaction between Ku70 subunit and HP1γ isoform. Such interaction promotes HP1γ Ser83 phosphorylation which overlaps exclusively with relaxed euchromatin and active RNA PolII transcription [106]. It is plausible that Ku70 and HP1γ association might also participate in DNA-PKcs activation and DSB repair.

8. DNA end processing enzymes

The simplest DSB is one that consists of two blunt DNA ends as these termini can be re-joined without processing. However, DSBs induced by ionizing radiation and reactive oxygen species are notorious for producing DNA ends which are non-ligatable ("dirty ends") and thus must be processed in order for ligation. Depending on the nature of the DNA break, different DNA end processing enzymes may be required, including those that resect DNA ends, fill in gaps, and remove blocking end groups, to create DNA ends that are ligatable. The long list of enzymes that the NHEJ pathway employs to process DNA ends underscores the versatility of the pathway. How specific enzymes are correctly chosen to fix each "dirty" end is not completely understood. Ku70/80 recruits, along with the canonical NHEJ factors, a large number of these processing enzymes to the DSB, which has resulted

in Ku being called a “tool-belt” protein [107]. The thought is that Ku recruits and stabilizes NHEJ factors which it deems required for the repair of each specific DSB [108]. This is supported by a study showing that the complexity of DNA damage influences the recruitment of NHEJ factors to the DSB [109]. Simple DSBs can be rapidly repaired by Ku70/80, XRCC4, DNA ligase IV, and XLF and complex breaks requiring those factors along with DNA-PKcs and possibly the activity of ATM. Collectively, this suggests the Ku heterodimer likely directs the utilization of specific subcomplexes to guide repair of individual DSBs. However, there is some belief that a fraction of NHEJ proceeds by a “trial and error” process in which each processing enzyme has an opportunity to fix the dirty end and that this process continues until the DSB is ligated and thus repaired [108].

A common incompatible end is one that contains a 3' or 5' single-strand overhang. These overhangs can be removed either by direct resection of the overhang or by using the nucleotide sequence of the overhang as a template to duplicate a complementary strand. The proteins implicated in resecting DNA ends for NHEJ include the endonucleases Artemis and Metnase, the RecQ helicase family member WRN, and aprataxin and PNKP-like factor (APLF). Artemis is the nuclease with the best established requirement for NHEJ. The confirmed activities of Artemis include a 5' endonucleases activity with a preference to nick a 5' overhang which leaves a blunt duplex end and the ability to remove 3'-phosphoglycolate groups from DNA termini [110, 111]. It should be noted that tyrosyl-DNA phosphodiesterase (Tdp1) can also repair 3'-phosphoglycolate-terminated DSBs and likely operates in NHEJ on special DSBs [112]. As stated above, the endonucleolytic activity of Artemis is regulated by DNA-PKcs and its phosphorylation status at the Thr2609 cluster [110]. Furthermore, Artemis can nick regions ssDNA within gaps in the double-stranded DNA and regions of mismatched bases which may be required for NHEJ [113]. Lastly, Artemis is required for the repair of complex DSBs and those that occur in the heterochromatin [114]. Metnase is an endonuclease that preferentially cleaves ssDNA and ssDNA-overhang of a partial duplex DNA, but Metnase appears to be not as efficient as Artemis in resolving DNA damaged ends in the presence of the NHEJ machinery [115, 116]. WRN and APLF have both been implicated in the NHEJ pathway. Both proteins have 3' to 5' exonuclease activity in vitro, but the activities of these two enzymes for processing DNA ends for NHEJ in vivo is still not clear [117–120].

The synthesizing of a complementary strand by adding nucleotides or filling of gaps of DNA for NHEJ is performed by the family X polymerases, which include the polymerases μ (Pol μ) and λ (Pol λ) and terminal deoxynucleotidyl transfer (TdT) [121]. These polymerase display a gradient of template dependency with TdT performing template-independent synthesis, Pol λ is nearly exclusively template-dependent, and Pol μ can carry out both [107, 121, 122]. These polymerases have no proofreading capability and are thus error-prone, but the gap filling activity of Pol λ is accurate in the presence of Ku and XRCC4/DNA Ligase IV [123]. Pol λ is tolerant of base damage, as it has been shown to have lyase activity [124]. Pol μ can catalyze DNA in the absence of complementary ends of the DNA via a unique end-bridging activity [125]. TdT is only found in lymphocytes and thus only plays a role in NHEJ during V(D)J recombination [126].

A number of factors have been shown to remove blocking end groups in order to make the termini of DSBs ligatable. A common non-ligatable end is one that contains either a 3' phosphate or 5' hydroxyl. The polynucleotide kinase/phosphatase (PNKP) is the NHEJ processing enzyme responsible for removing 3' phosphate and 5' hydroxyl groups and restoring the ligatable 3' hydroxyl and 5' phosphate groups. PNKP performs this function via its 5' kinase and 3' phosphatase activities [127]. The removal of adenylate groups covalently linked to 5' phosphate termini are removed by Apratxin, which is a member of the histidine triad family of nucleotide hydrolases and transferases [128]. A surprising discovery was that Ku is able to excise abasic sites near the DSB via an intrinsic 5'deoxyribose-5-phosphate (5'-dRP)/AP lyase activity [129]. Abasic sites within a short 5' overhang at the DSB end were found to be removed best by Ku.

9. XRCC4, DNA Ligase IV, and XLF

The final step in the repair of a DSB via NHEJ is ligation of the broken ends, which is mediated by DNA LigaseIV. DNA ligase IV has activity on its own, but its activity is stimulated by XRCC4 via the ability of XRCC4 to stabilize DNA LigaseIV and promote its adenylation [130]. DNA Ligase IV is a flexible ligase that can ligate incompatible DNA ends and ligate across gaps [131]. XLF facilitates the ability of DNA Ligase IV to ligate mismatched and non-cohesive ends and primes DNA Ligase IV for its next catalytic activity by promoting its readenylation [132–135]. Recently, it was shown that APLF stimulates ligation by XRCC4-DNA LigaseIV above that observed in the presence of only Ku70/80 [136]. APLF also was shown to play a role in assembly of the NHEJ complex and that its main function, in conjunction with PARP3, may be to promote the retention of XRCC4, DNA LigaseIV, and XLF at DSBs and promoting faster ligation [136, 137].

The versatility of the NHEJ pathway and its factors has been greatly supported by the recent reports that show that XRCC4, DNA Ligase IV, and XLF have functions that appear to be independent of the terminal ligation step of the pathway. Interestingly, DNA bound Ku70/80 may not be the only NHEJ protein mediating recruitment of the NHEJ machinery to the DNA damage site as XRCC4 assists in the recruitment of the NHEJ-dependent DNA end processing enzymes to DSBs and may play a role in the ability of the NHEJ complex to choose the correct enzymes to aid in the repair of a specific break. XRCC4 interacts alone or in conjunction with Ku70/80 with the following DNA end processing enzymes, DNA ligase IV, DNA polymerase μ [138], WRN [117, 119, 139], PNKP [140], aprataxin [141], and APLF [120, 142, 143]. Furthermore, XRCC4 may also function in bridging DNA ends. XRCC4 directly interacts with the globular head domain of XLF, creating a head to head interface between the two proteins and structural analysis found that XRCC4 and XLF form super helical filaments via alternating XRCC4 and XLF head domain interfaces which are able to interact with DNA and bridge broken DNA strands [144–147]. But, it should be noted that the relevance of the XRCC4-XLF multimers in vivo is still under debate as addition of the catalytic domain of DNA Ligase IV destabilizes the XRCC4-XLF multimeric complex [148]. However, in human cells XRCC4 is approximately six-fold excess relative to DNA Ligase IV suggesting that XRCC4 likely exists in complexes independently of DNA Ligase IV in vivo [149]. Finally, it has been reported that the ligation complex exhibits a non-catalytic function in NHEJ by promoting DNA-PKcs-mediated DNA end synapsis and

DNA-PKcs autophosphorylation [26]. This data suggests that a productive NHEJ supramolecular complex consisting of all the canonical NHEJ factors may be responsible for protecting DNA ends.

10. Conclusions

In this review we described the NHEJ and the important factors and mechanisms that regulate this DSB repair pathway. The working model for NHEJ has continued to grow more complex. NHEJ is a versatile repair process and growing evidence suggest that there may be subcomplexes within the NHEJ repair pathway that are required for the repair of specific DSBs. These insights have opened up a number of new and unresolved questions including: (1) what factors are required for the repair of each type of DSB and what mechanism regulate the choice of these specific factors; (2) what is the role does the kinase activity of DNA-PKcs play in NHEJ and what are its relevant substrates that are required for NHEJ; and (3) what factors and specific mechanisms are required for the dissolution of the NHEJ complex once repair is completed?

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Abbreviations

DSBs	DNA double strand breaks
NHEJ	non-homologous end-joining
HR	homologous recombination
DNA-PK	DNA dependent protein kinase
Ku	Ku70/80
DNA-PKcs	DNA dependent protein kinase catalytic subunit
XRCC4	X-ray cross complementing protein 4
XLF	XRCC4-like factor
PI3K	phosphatidylinositol-3 kinase
PIKK	phosphatidylinositol-3 kinase-like kinase
ATM	ataxia-telangiectasia mutated
ATR	ATM and Rad3-related
HEAT	Huntington-elongation-A-subunit-TOR
FAT	FRAP, ATM, TRRAP
FATC	FAT C-terminal
WRN	Werner

Thr2609	threonine 2609
Ser2056	serine 2056
APLF	aprataxin and PNKP-like factor
PNKP	polynucleotide kinase/phosphatase
EGFR	epidermal growth factor receptor
EGF	epidermal growth factor
IR	irradiation
HP1	heterochromatin protein 1
CK2	casein kinase II

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Highlights

- A review of the DNA double strand break repair pathway non-homologous end-joining is provided.
- The important proteins and mechanisms modulating non-homologous end-joining are highlighted.
- Emphasis is given to the DNA-PK complex and the versatility of non-homologous end-joining for the repair of DNA double-stranded breaks.

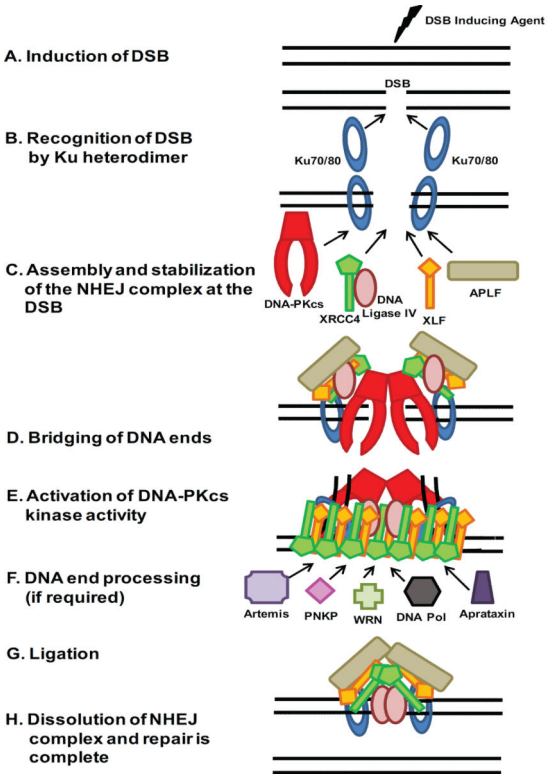

Figure 1.

Schematic overview of NHEJ. Diagram outlines each basic step of the NHEJ pathway along with the proteins implicated for each step. It should be noted that although shown in this figure, the multimerization of XRCC4 and XLF is still controversial and that a number of factors, such as DNA-PKcs and Artemis, may exist as pre-existing binary complexes as early as step IV. See text and [7] for details.

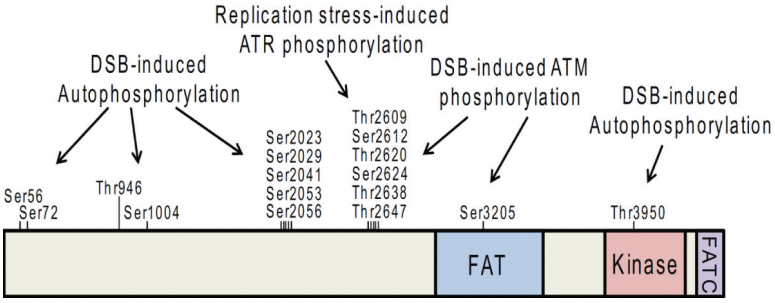

Figure 2.

Phosphorylation sites within the DNA-PKcs protein. The best characterized phosphorylation sites are indicated. The phosphorylation clusters (Thr2609 and Ser2506) and individual phosphorylation sites are described in full detail in the text.