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Non-homologous end joining: emerging themes and unanswered questions

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Abstract

Non-homologous end joining (NHEJ) is the major pathway for the repair of ionizing radiation induced DNA double strand breaks in human cells. Here, we discuss current insights into the mechanism of NHEJ and the interplay between NHEJ and other pathways for repair of IR-induced DNA damage.

Keywords

Non-homologous end joining; ionizing radiation; DNA double strand break repair

1. Introduction

Non-homologous end joining (NHEJ) is the major pathway for the repair of ionizing radiation (IR)-induced DNA double strand breaks (DSBs) in human cells and is essential for the repair of RAG-induced DSBs during V(D)J recombination [1-3]. Evidence is emerging that aberrant NHEJ is also a major source of genomic rearrangements and chromosomal translocations [4], leading to genomic instability, a hallmark of cancer [5]. Proteins shown to be required for NHEJ and V(D)J recombination in mammalian cells include the Ku70/80 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis, XRCC4, DNA ligase IV and XLF (XRCC4-like factor, also called Cernunnos). Loss of any of these core NHEJ factors is associated not only with radiation sensitivity but with defective V(D)J recombination and, in animals and patients, immune defects [1, 3, 6-11].

Much of what we know about the mechanism of NHEJ combines results from studies on the cellular responses to IR and other DNA damaging agents as well as studies on the mechanism of V(D)J recombination. V(D)J recombination occurs at discrete sites (recombination signal sequences) in immunoglobulin, T cell receptor and B cell receptor genes, and produces closed DNA hairpin ends (at the coding ends) that upon hairpin

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opening, processing and rejoining go on to form the coding sequence, and 5'-phosphorylated blunt signal ends that are sealed and lost from the system [3]. In contrast, IR induced lesions are produced stochastically and thought to be dispersed randomly throughout the genome. Also, due to the nature of IR-induced DNA damage, IR-induced DSBs are inherently complex and variable in nature. A characteristic of IR-induced DNA damage is damage or loss of bases, as well as production of DNA single strand breaks (SSBs) that frequently terminate in non-ligatable end groups such as 3'-phosphates, 3'-phosphoglycolates, or 5'-hydroxyl groups [12, 13]. The presence of two SSBs on opposite strands of the DNA approximately 10 bp apart yields a DSB, therefore IR-induced DSBs have short overhanging ends that terminate in non-ligatable end groups that must be processed or removed prior to ligation (reviewed in [13]). Moreover, IR-induced DSBs are frequently surrounded by other DNA lesions, such as base damage, that together make IR-induced DSBs difficult to repair and one of the most cytotoxic forms of DNA damage [13]. Consequently, NHEJ in response to IR-induced DNA damage requires flexibility to respond to complex and variable types of DSBs.

For simplicity, we can think of NHEJ of IR-induced DSBs as proceeding in three distinct stages. First is detection of the DSB by the Ku70/80 heterodimer and tethering of the DNA ends, by the Ku - DNA-PKcs complex. Second, is processing of the damaged DNA termini by a variety of enzymes including polynucleotide kinase phosphatase, PNKP, as well as gap-filling DNA polymerases μ and λ . Lastly, is rejoining of the DSB ends by the XRCC4-DNA ligase IV complex [2, 14] (Fig. 1). However, apart from DSB detection by Ku, the order of recruitment of the different NHEJ proteins maybe quite flexible, and not all NHEJ components may be required for repair of all lesions [15, 16]. Thus, a model is emerging in which NHEJ may take place within a dynamic, multi-protein complex rather than in a stepwise, more sequential manner in which one component is released before another is recruited. In this review, we will discuss current thinking on the mechanism of NHEJ of IR-induced DSBs and speculate on how NHEJ and other repair pathways interact to repair IR-induced DNA damage throughout the cell cycle.

2. The Ku heterodimer - a multi-functional NHEJ protein

IR-induced DSBs are detected by the abundant, nuclear protein Ku70/80, composed of approximately 70 and 80 kDa subunits, that binds double stranded (ds) DNA ends with high affinity in a largely sequence independent manner [17]. Ku70 and Ku80 form a heterodimer with a preformed ring that encircles ends of dsDNA [18], making it ideally suited for binding to DSB ends. However, in recent years it has become apparent that the Ku70/80 heterodimer plays other critical roles in NHEJ, not only in the initial detection of the DSB but in the recruitment of essentially all of the core NHEJ factors to the DSB. Ku is required for recruitment of DNA-PKcs to DSBs [19] and stimulation of DNA-PKcs kinase activity [20]. Since DNA-PKcs interacts with Artemis [21], Ku may also be responsible, indirectly, for the recruitment of Artemis to DSBs. Ku80 also interacts with XLF, and Ku is required for recruitment of XLF to DSBs in vivo [22, 23]. Moreover, Ku70 interacts with XRCC4 and laser micro-irradiation studies suggest that Ku enhances retention of XRCC4 at DSBs [24]. In addition, Ku80 interacts with APLF (aprataxin and PNKP-like factor, also called PALF for PNKP and aprataxin-like factor), which enhances NHEJ, stimulating DSB end

ligation by DNA ligase IV [25, 26]. Ku also interacts with the Werner's syndrome helicase [27] as well as with DNA polymerase mu and lambda [28, 29] suggesting roles in the recruitment or retention of additional end-processing factors at the break (Fig. 1).

3. Processing complex DNA damage at DSB ends

As discussed above, IR induced DSBs are considered complex, frequently containing base damage close to the DSB as well as short DNA overhangs and non-ligatable DNA termini [12, 13]. Accordingly, several enzymes have been implicated in DSB end-processing in NHEJ, but which enzymes are involved likely depends on the nature of the DNA damage incurred. In addition to its critical roles in DSB detection and recruitment of other NHEJ factors, Ku has recently been shown to possess 5'-RP/AP lyase activity, suggesting that it also plays a catalytic role in NHEJ, modifying damaged bases proximal to the DNA ends [30, 31]. PNKP has 3'-DNA phosphatase and 5'-DNA kinase activities, making it an ideal candidate for removing IR-induced non-ligatable DNA end groups at DSB termini [32]. PNKP interacts with scaffolding protein XRCC4 in a phosphorylation-dependent manner involving the N-terminal fork-head associated (FHA) domain of PNKP and CK2-Thr233 phosphorylated XRCC4 suggesting a mechanism by which it is recruited to DSBs [33]. The FHA domain of PNKP also interacts with CK2-phosphorylated XRCC1, conferring roles in repair of IR-induced SSBs as well as DSBs [34]. Accordingly, PNKP null cells are radiation sensitive, consistent with a role in repair of IR-induced strand breaks [35].

Recently, two other proteins have been identified that bear a PNKP-like FHA domain: APLF, and aprataxin (APTX). Like PNKP, APLF interacts with CK2-phosphorylated XRCC4 and XRCC1 via its N-terminal FHA domain [36, 37]. APLF also interacts directly with Ku80, through its central domain [25, 26] and enhances NHEJ and DNA ligase IV mediated ligation in NHEJ [25, 38]. Moreover, APLF has been reported to have nuclease activity, suggesting possible roles in DSB end processing [36, 39]. APLF also interacts with poly-ADP ribose and histones, providing links between NHEJ and chromatin [40, 41].

In contrast, APTX removes AMP from the 5'-termini of DNA breaks that result from abortive ligation events [42]. Like PNKP and APLF, APTX interacts with phosphorylated XRCC4 and XRCC1 [43]. Absence of APTX confers defects in DNA SSB repair and is associated with the neurological condition, ataxia with oculomotor apraxia 1 (AOA1) [44], however, to date, no defects in DSB repair have been reported (reviewed in [45]). Factors that regulate the ability of phosphorylated XRCC4 (and XRCC1) to interact with APLF, APTX and PNKP remain to be elucidated.

Additional end processing in NHEJ is carried out by DNA polymerases mu and lambda (and TdT in V(D)J recombination) which interact with Ku and the XRCC4-DNA ligase IV complex and perform gap filling roles [29]. Artemis, a structure specific endonuclease interacts with DNA-PKcs and plays a critical role in opening DNA hairpin coding ends in V(D)J recombination [21, 46]. Artemis was originally suggested to have exonuclease activity [21], but this has recently been questioned [47]. Artemis null cells are mildly radiation sensitive suggesting that it is required for repair of a subset of IR-induced DSBs [48], however, its DNA substrates in the DNA damage response are not known.

V(D)J recombination assays reveal that DNA-PKcs phosphorylation plays an important role in regulating the access of nucleases to DNA ends prior to ligation. DNA-PKcs is recruited to DSBs by Ku, and the protein kinase activity of DNA-PKcs is required for NHEJ [49, 50]. DNA-PKcs phosphorylates multiple NHEJ proteins in vitro [2, 51-53] and autophosphorylates on multiple sites in vitro [54]. DNA-PKcs is also highly phosphorylated in vivo (reviewed in [55]). The most well characterized phosphorylation sites in DNA-PKcs, located between threonines 2609 and 2647 are termed the ABCDE cluster [56, 57]. These sites are phosphorylated in a DNA-PK-dependent manner in vivo (consistent with autophosphorylation) [58, 59], but can also be phosphorylated by other members of the phosphatidylinositol-3 kinase-like protein kinase (PIKK) family, ataxia telangiectasia mutated (ATM) and ATM, Rad3-related (ATR) (reviewed in [2, 53, 55]). Ablation of three of the ABCDE sites in mice (corresponding to threonines 2609, 2638 and 2647 in human DNA-PKcs) results in severe bone marrow failure and lethality [60]. DNA-PKcs also autophosphorylates on serine 2056, located in the PQR cluster, in response to DNA damage [58, 59]. Ablation of ABCDE and PQR phosphorylation sites within DNA-PKcs affects the length of DSB end termini in a reciprocal manner [61] suggesting that phosphorylation of different sites within DNA-PKcs regulates accessibility of the DNA termini to nucleases (reviewed in [2, 53, 62]). DNA-PKcs phosphorylation has also been shown to play an important role in regulating homologous recombination repair (HRR) [63, 64] (discussed in more detail below). DNA-PKcs dependent regulation of both end processing and pathway choice has been proposed to occur through phosphorylation-dependent conformational changes that result in dissociation of DNA-PKcs from the DNA-Ku complex [55, 65]. Interestingly, a recent study suggests that DNA-PKcs is only required for the repair of complex DSBs i.e. DSBs which are surrounded by additional DNA lesions [66], consistent with the theme of flexibility within the NHEJ pathway. Also, DNA-PKcs is not found in yeast or in most lower multicellular eukaryotes (with the exception of *Dictyostelium* and possibly *Ciona* [67]), consistent with it being required for repair of a subset of breaks, and/or those in the genomes of a subset of eukaryotes.

4. Sealing the break

Once DNA ends have been processed, they are covalently joined by DNA ligase IV, which exists in complex with XRCC4, which, in turn, interacts with XLF [68]. The XRCC4 homodimer interacts with the BRCT domains of DNA ligase IV with high affinity via its coiled coil stalk domain [69, 70] and, with lower affinity, with the XLF homodimer [71]. XRCC4 homodimers can also interact with themselves, to form higher order complexes, and with dsDNA (reviewed in [72]). XRCC4 is required for stabilization of DNA ligase IV and stimulates its activity [73]. In vitro studies suggest that XLF is only required for repair at a subset of breaks, involving mismatched or non-cohesive ends [74]. XLF also stimulates the re-adenylation activity of DNA ligase IV, which is essential for re-charging DNA ligase IV, allowing a new catalytic cycle [71]. In vitro studies reveal that XRCC4 and XLF interact via their head domains to form long helical protein filaments that may be involved in bridging DNA ends or holding DNA ends in an orientation that facilitates ligation [75-78]. How DNA ligase IV and other XRCC4 interacting proteins such as PNKP and APLF have direct

access to DSB ends within the context of XRCC4-XLF filaments remains to be determined (reviewed in [72]).

5. A Ku-dependent NHEJ complex?

Given the requirement of DNA ligase IV for ligation of the DNA, it has long been assumed that the XRCC4-DNA ligase IV complex functions in the final stage of NHEJ, however recent studies have revealed that DNA ligase IV deficient cells are unable to support phosphorylation of DNA-PKcs, suggesting that DNA-ligase IV is required for DNA-PKcs' activation and/or the function of DNA-PKcs at DSB ends [79]. These findings suggest that the XRCC4-DNA ligase IV complex assembles with Ku and DNA-PKcs at DSBs, which in turn suggests that Artemis (recruited by DNA-PKcs), XLF and APLF (recruited by Ku and XRCC4) and PNKP (thought to be recruited by XRCC4) may also be recruited to the DSB to form a multi-protein complex. DNA-ligase IV (which interacts with XRCC4 and is recruited to Ku at DSBs) also interacts with Artemis (which in turn interacts with DNA-PKcs) and this interaction is required for V(D)J recombination [80, 81]. Thus, a picture is emerging of multiple proteins, recruited as needed, in a Ku-dependent manner to the DSB and possibly stabilized by multiple additional protein-protein and protein-DNA interactions.

This model then raises further questions regarding how individual proteins within this putative complex would gain access to the actual DSB ends. One possibility is that DNA-PKcs-mediated autophosphorylation, phosphorylation of other proteins and resulting conformational changes remodel the NHEJ complex, regulating appropriate and timely access of NHEJ proteins to the DNA ends [53, 62]. It is also worth noting that DNA damage-induced phosphorylation of several NHEJ proteins, including Artemis [82-84], XLF [85], PNKP [86, 87], APLF [88-90] as well as some phosphorylation sites on DNA-PKcs [91, 92] requires ATM rather than DNA-PKcs. Since ATM is required for NHEJ at complex lesions and/or at DSBs in heterochromatic regions [93, 94], this suggests that ATM-dependent phosphorylation of NHEJ proteins may regulate NHEJ at a subset of DSBs.

6. Competition between NHEJ and other DNA repair and DNA damage response pathways

NHEJ is active throughout the cell cycle, whereas repair of DSBs via HRR repair occurs only in late S and G2 when a sister chromatid is available for accurate, template-mediated repair [95]. These observations raise important questions regarding pathway choice: specifically, how Ku and the MRN complex (composed of Mre11, Rad50 and Nbs1), compete for detection of DSBs in different stages of the cell cycle. For example, DSB detection by Ku would channel repair into NHEJ in all stages of the cell cycle, whereas, DSB detection by MRN channels repair into HRR in G2 but additionally, results in activation of ATM throughout the cell cycle (Fig. 2). Initiation of HRR requires the generation of long regions of ssDNA that are detected initially by RPA and subsequently bound by Rad51 to allow strand invasion [96]. Therefore, factors that control cell cycle associated DNA end resection are critical to the choice between NHEJ and HRR in G2 (discussed in detail in [97] and [98]). Interestingly, the nuclease activity of Mre11 is critical

for end resection and initiation of HRR but not for activation of ATM [99, 100] revealing distinct structural and catalytic roles for the MRN complex in regulating pathway choice.

Recent studies suggest that DNA-PKcs and Ku play important roles in regulating the balance between NHEJ and HRR in cells. Loss of either Ku or DNA-PKcs results in enhanced DNA end resection in G2 phase cells, which consequently promotes repair by HR [63]. Moreover, inability of DNA-PKcs to undergo autophosphorylation at the ABCDE cluster causes delay in Rad51 foci formation [63]. One explanation for these observations is that abrogation of DNA-PKcs' ability to undergo autophosphorylation at the ABCDE sites prevents or delays its release from DSB ends, thus delaying initiation of HRR [63]. From these studies, Jeggo and colleagues suggest that NHEJ attempts to repair IR-induced DSBs in all stages of the cell cycle. If successful, the DSB is repaired rapidly. In G2, if NHEJ is unsuccessful, resection ensues, committing the cell to HRR [63, 97].

In vitro studies from the Paull laboratory demonstrate that the presence of DNA-PKcs blocks Exonuclease 1 (Exo1) activity and that DNA-PKcs' autophosphorylation or phosphorylation of DNA-PKcs by ATM, is required to relieve inhibition of Exo1 by DNA-PKcs [101], again consistent with autophosphorylation of DNA-PKcs being required for its release from DNA ends and subsequent pathway progression [55]. Interestingly, the authors also showed that the MRN complex promotes DNA-PKcs autophosphorylation in vitro [101], raising the interesting possibility that MRN may facilitate removal of DNA-PKcs from DNA ends, to facilitate HRR.

Another significant recent development has been the generation by the Tainer laboratory of small molecule inhibitors specific for Mre11 endo- or exonuclease activities [99]. Mre11 nuclease activity is critical for initiation of HRR but not for activation of ATM [100]. Through the use of Mre11 endo- and exo-nuclease specific inhibitors, Tainer and Jeggo propose a two-step process in which the endonuclease activity of Mre11 first introduces a nick in the 5' strand, upstream of the DSB, which is followed by bi-directional resection utilizing Mre11 3'-5' exonuclease activity in concert with the 5'-3' exonuclease activity of the Exo1/BLM complex [99].

Another important regulator of pathway choice is 53BP1, which, with its interacting partner Rif1, acts to promote NHEJ in G1 by protecting DSB ends from DNA end-resection [102-106] (Fig. 2). Elucidation of how 53BP1 carries out this important function however, the mechanism by which it achieves this is unclear. One of the obstacles to our ability to address pathway choice has been the inability to detect Ku at DSBs in cells. The recent development of methods to visualize individual Ku heterodimers at DSB termini may afford new ways to directly address the relative positioning and timing of Ku, MRN, 53BP1 and other DNA damage sensing proteins at DSBs in cells [107].

Since IR induces damage to DNA bases as well as DNA single strand breaks, much also remains to be learned about how NHEJ interfaces with base excision repair and DNA single strand break repair pathways as well as alternative NHEJ pathways [108, 109]. Since DSBs are regarded to be more toxic than single strand breaks or base lesions, it seems probable that DSBs would be repaired first with high priority, whereas repair of base lesions and

single strand breaks in proximity to the DSB may be repaired more slowly, once the DSB ends have been ligated. Interestingly, however, base damage upstream of a DSB has been shown to impair NHEJ [110], thus IR-induced base damage may directly impinge on the ability NHEJ to repair IR-induced DSBs. Unraveling the complexities of pathway choice will clearly continue to be a major focus for future investigations.

7. Concluding remarks

It has been over twenty years since DNA-PKcs was identified [111, 112] and Ku and DNA-PKcs were shown to form the DNA-PK complex [20, 113, 114]. Evidence for their involvement in DSB repair quickly followed [115–118]. The discovery of additional components of the NHEJ pathway, such as XRCC4 [119], the XRCC4-DNA ligase IV complex [120], Artemis [48] and XLF [68, 121] followed and our understanding of the mechanisms of DSB repair in eukaryotes as well as V(D)J recombination has increased dramatically in past decades (reviewed in [3, 122]). Recent findings, described here, have begun to reveal how these proteins interact with each other and with their target, damaged DNA. Significant progress is also being made in elucidating the structural biology of individual NHEJ components, and pictures of NHEJ interactions in multi-protein-DNA complexes is beginning to emerge (reviewed in [123]). As we embark upon the third decade of research into NHEJ, there is still much to learn about how the NHEJ complex is regulated, how it interfaces with chromatin and other DNA damage response pathways and how these pathways can be targeted for therapeutic advantage in cancer therapy.

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Figure 1. A model for NHEJ illustrating the central role of the Ku70/80 heterodimer
Adapted from [14] with permission. DSBs are detected by the Ku70/80 heterodimer, which interacts with multiple components of the NHEJ machinery, including DNA-PKcs, XLF, WRN and APLF, recruiting them to DSBs. Once the DSB is detected, various enzymes may be involved in processing of the DSB ends, for example, removal of non-ligatable end groups by PNKP, and gap filling by DNA polymerases mu and lambda. The break is resealed by DNA ligase IV, which exists in complex with XRCC4 and XLF. Recent studies suggest that the XRCC4-DNA ligase IV complex is required for DNA-PKcs autophosphorylation [79], suggesting that XRCC4 and DNA ligase IV may be present at the break as a multi-protein complex rather than recruited in the later stages of NHEJ. How Ku is removed from DSBs is unknown, but may involve proteolytic degradation [124-126] or Mre11 nuclease activity [99]. Direct protein-protein interactions are indicated by black lines. The AP lyase activity of Ku [30, 31] is shown by the red arrow. ATM and DNA-PK dependent phosphorylation events are shown in red and blue P symbols respectively. See text and [14] for details.

Figure 2. A simplified model for the cellular response to IR-induced DNA damage
Adapted from [14] with permission. IR induces base damage and SSBs. Two SSBs on opposite strands of the DNA induce a DSB. Damaged, non-ligatable termini are represented by the small red circles. The DSB can be detected by the Ku heterodimer, initiating NHEJ, the major pathway for the repair of IR-induced DSBs, which is active in all stages of the cell cycle. In the absence of classical NHEJ, an alternative or back-up NHEJ pathway, that involves PARP1, XRCC1 and DNA ligases I or III may be initiated [127]. DSBs are detected by the Mre11, Rad50, Nbs1 (MRN) complex which leads to activation of the ATM protein kinase and downstream signaling events that induce cell cycle checkpoint arrest and other cellular responses to DSBs [128]. The MRN complex is required for 5'-3' resection, likely involving Mre11, CtIP, Exo1, Dna2 and other proteins, which produces long 3' ends that are recognized by ssDNA-binding protein, RPA. The 53BP1/RIF complex promotes NHEJ by blocking DSB ends from resection [102-106], but the mechanism by which it does so is poorly understood. RPA is displaced by BRCA2, allowing binding of Rad51 and initiation of homologous recombination repair (HRR), which is active only in late S and G2 phases of the cell cycle [96, 129]. Recruitment of RPA also initiates activation of ATR,

which like ATM, contributes to activation of cell cycle checkpoints and other cellular responses [130]. Precisely how Ku and MRN compete or cooperate to initiate NHEJ, HRR and activation of ATM is an area of active investigation. See text for details.