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Repair of ionizing radiation-induced DNA double strand breaks by non-homologous end-joining

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Abstract

DNA double strand breaks (DSBs) are considered the most cytotoxic type of DNA lesion. They can be introduced by external sources such as ionizing radiation (IR), by chemotherapeutic drugs such as topoisomerase poisons and by normal biological processes such as V(D)J recombination. If left unrepaired, DSBs can cause cell death. If misrepaired, DSBs may lead to chromosomal translocations and genomic instability. One of the major pathways for the repair of IR-induced DSBs in mammalian cells is non-homologous end-joining (NHEJ). The main proteins required for NHEJ in mammalian cells are the Ku heterodimer, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), Artemis, XRCC4, DNA ligase IV and XLF (XRCC4-like factor, also called Cernunnos). Additional proteins including DNA polymerases μ and λ , polynucleotide kinase (PNK) and the Werner's Syndrome helicase (WRN) may also play a role. Here, we will review our current understanding of the mechanism of NHEJ in mammalian cells and discuss the roles of DNA-PKcs and DNA-PK-mediated phosphorylation in NHEJ.

Keywords

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

INTRODUCTION

DNA double strand breaks (DSBs) are considered the most lethal form of DNA damage. They can be introduced by exogenous agents such as ionizing radiation (IR), topoisomerase poisons, radiomimetic drugs (*e.g.* bleomycin and neocarzinostatin), and by cellular processes such as V(D)J recombination, class switch recombination, stalled replication forks and reactions that generate reactive oxygen species [1,2]. In this review, we will focus on the detection and repair of IR-induced DSBs by the non-homologous end-joining (NHEJ) pathway.

All organisms are exposed to low doses of naturally occurring IR, and IR is widely used in medical procedures such as X-rays and radiation therapy for the treatment of cancer patients [3–5]. IR damages DNA by direct deposition of energy and also indirectly, by ionization of water molecules to produce hydroxyl radicals that attack the DNA. IR induces multiple forms of DNA damage including damage to the bases, and cleavage of the DNA backbone to form DNA single strand breaks (SSBs). These types of DNA damage are detected and repaired by

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the base excision repair (BER) and SSB repair pathways, respectively [6,7]. DSBs are formed when two SSBs occur on opposite DNA strands approximately 10–20 bp apart. Thus, IR-induced DSBs usually contain overhanging 3' and 5' ends. In addition, the DNA termini frequently contain 3'-phosphate or 3'-phosphoglycolate groups, which must be removed prior to ligation [8] (Figure 1 (A)). Moreover, the DNA surrounding the DSB may contain additional forms of DNA damage, producing what are termed complex or clustered lesions [9]. If not repaired, such lesions can result in cell death. If misrepaired, DSBs have the potential to result in chromosomal translocations and genomic instability [1,10].

In mammalian cells there are two major pathways for the repair of IR-induced DSBs, namely NHEJ and homologous recombination or homology directed repair (HDR) [2,4]. HDR is an accurate form of repair, which requires an undamaged sister chromatid to act as a DNA template and functions only after DNA replication [2,11]. In contrast, NHEJ is active throughout the cell cycle [12] and is considered the major pathway for the repair of IR-induced DSBs in human cells [11]. In its simplest sense, NHEJ entails straightforward ligation of DNA ends. However, since the DNA ends formed by IR are complex and frequently contain non-ligatable end groups and other types of DNA damage, successful repair of DNA lesions by NHEJ must require processing of the ends prior to ligation. This can lead to loss of nucleotides from either side of the break, making NHEJ potentially error prone. In addition to HDR and NHEJ, there is also increasing evidence for the existence of alternative end-joining pathways that directly ligate DNA ends in the absence of NHEJ [13–17]. However, whether these pathways function in normal cells or only when NHEJ is deficient is not clear.

THE NHEJ PATHWAY IN MAMMALIAN CELLS

NHEJ in mammalian cells requires the Ku70/80 heterodimer, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), XRCC4, DNA ligase IV, Artemis, and XLF (XRCC4-like factor, also called Cernunnos). Deletion or inactivation of any of these core NHEJ factors induces marked sensitivity to IR and other DSB inducing agents as well as defects in V(D)J recombination [18–21]. Increasing evidence suggests that additional DNA processing enzymes such as DNA polymerases μ and λ , polynucleotide kinase (PNK) and the Werner's syndrome helicase (WRN) also play a role in NHEJ, at least at a subset of DNA ends. In general terms, NHEJ is thought to proceed through the following stages: (1) detection of the DSB and tethering/protection of the DNA ends; (2) DNA end-processing to remove damaged or non-ligatable groups, and (3) DNA ligation. In the following sections, we will review the roles of the main players in NHEJ in each of these steps and propose a model for how they may function in NHEJ. Since the protein kinase activity of DNA-PKcs is required for NHEJ [22,23], we will also discuss the role of DNA-PK-mediated phosphorylation in the process.

Detection of the DSB and tethering of the DNA ends

The Ku70/80 heterodimer—The first step in NHEJ is detection of the DSB by the Ku70/80 heterodimer (Ku) (Figure 1(B)). Ku is composed of Ku70 and Ku80 subunits, which each contribute to a central DNA binding core [24]. In addition, the N- and C-terminal regions of Ku70 and Ku80 contain unique regions. The N-terminus of Ku70 contains an acidic domain that is phosphorylated *in vitro* by DNA-PKcs [25]; while the C-terminus contains a SAP (SAF-A/B, Acinus and PIAS) domain which is a putative chromatin/DNA binding domain [26] (Figure 2A). The C-terminal region of Ku80 forms a long flexible arm that may be involved in protein-protein interactions [27, 28], and, at the extreme C-terminus, a conserved region which is required for interaction with DNA-PKcs [29–31] (Figure 2B). *In vitro*, Ku binds to ends of double-stranded (ds) DNA with high affinity and without apparent sequence specificity [32]. This property is due to the structure of the Ku70/80 DNA binding core, which adopts a pre-formed loop that encircles the DNA [24] (Figure 1(B)). Binding of Ku to the DSB ends may assist in tethering the broken ends together [33]. Once bound, Ku translocates inwards

from the DNA end (Figure 1(C)) making the extreme termini accessible to other proteins, such as DNA-PKcs [34] (Figure 1(D), and described in detail below).

Recent studies using laser microbeam irradiation to induce DNA damage in the nuclei of living cells have shed light on the order of recruitment of NHEJ factors to sites of DNA damage as well as the kinetics of the repair process. Consistent with Ku being the major DSB sensing protein in NHEJ, fluorescently-tagged Ku localizes to sites of laser-induced DNA damage in cells within a few seconds and independently of other NHEJ or DSB repair proteins [35,36]. Recruitment of Ku to the DSB also serves to recruit other NHEJ proteins to the DSB. As discussed below, Ku interacts with DNA-PKcs (reviewed in [26,37]), the XRCC4-DNA ligase IV (X4-L4) complex [36,38,39], XLF [40], DNA pol μ [41] and DNA pol λ [42] *in vitro*. The interactions of DNA-PKcs [43], XLF [44], DNA pol μ [42] and DNA ligase IV [38] with Ku are facilitated by or enhanced in the presence of DNA, suggesting that binding of Ku to DNA is a prerequisite for interaction with other NHEJ proteins. Interestingly, binding of Ku to DNA results in a conformational change in the flexible C-terminal regions of both Ku70 and Ku80, which might facilitate its interactions with partner proteins [45]. Ku is also required for the recruitment of DNA-PKcs [46], XRCC4 [36] and XLF [40] to sites of DNA damage *in vivo*. Thus, Ku can be regarded as the cornerstone of NHEJ.

DNA-PKcs—One of the first proteins shown to interact with Ku was DNA-PKcs. DNA-PKcs, the product of the *PRKDC* gene, is a large polypeptide of over 4000 amino acids, and a member of the phosphatidylinositol 3-kinase like family of protein kinases (PIKKs) (reviewed in [26,47]). The amino terminal ~250 kDa of DNA-PKcs contains a putative DNA binding domain [48], a leucine rich region and a series of HEAT (*huntingtin, elongation factor 3, A* subunit of protein phosphatase 2A and *TOR1*) repeats [49] but few other distinguishing features (Figure 3). The C-terminal region contains a FAT (*FRAP, ATM, TRRAP*) domain that is characterized by weak amino acid similarity to other members of the PIKK family, followed by a kinase domain and a C-terminal FATC domain (Figure 3). Cells that lack DNA-PKcs are highly radiosensitive and have defects in V(D)J recombination, specifically in processing of coding joints. Moreover, in mice, dogs and horses, DNA-PKcs deficiency is associated with severe combined immunodeficiency (SCID) (reviewed in [20,21]).

The interaction between DNA-PKcs and Ku is mediated by a conserved region in the extreme C-terminus of Ku80 [29–31] (Figure 2B), and C-terminal regions of DNA-PKcs have been implicated in its interactions with Ku [50,51] (Figure 3). Ku and DNA-PKcs only interact in the presence of DNA [52] and recruitment of DNA-PKcs to sites of DNA damage *in vivo* is Ku-dependent [46]. Inward translocation of Ku allows DNA-PKcs to interact with the extreme termini of the DNA [34], allowing two DNA-PKcs molecules to interact across the DSB in a so-called “synaptic complex” [53] (Figure 1(D)). This interaction stimulates the kinase activity of DNA-PKcs [53], promoting phosphorylation *in trans* across the DSB [54] (discussed in more detail below). Once assembled at the DNA ends, the DNA-PKcs-Ku-DSB complex serves to tether the ends of the DSB together and protects the DNA ends from nuclease attack.

The protein kinase activity of DNA-PKcs—Alone, DNA-PKcs has weak serine/threonine kinase activity that is greatly enhanced in the presence of dsDNA ends and Ku [43]. The DNA-PKcs-Ku-DNA complex is referred to as DNA-PK (Figure 1(D)). Like other members of the PIKK family, DNA-PK phosphorylates many of its substrates on serines or threonines that are followed by glutamines (SQ/TQ motifs) [55,56], however, DNA-PK also phosphorylates proteins on non-SQ/TQ sites *in vitro* [25,57–59]. Significantly, the protein kinase activity of DNA-PKcs is required for NHEJ [22,23], therefore, identification of its physiological targets is critical to understanding its function in NHEJ. Moreover, inhibitors of DNA-PK kinase activity radiosensitize cells and inhibit DSB repair, making DNA-PK a possible therapeutic target [60,61].

Given its role in NHEJ, obvious candidates for physiological substrates of DNA-PKcs are other NHEJ factors. However, although DNA-PK phosphorylates Ku70, Ku80, XRCC4, XLF, Artemis, and DNA ligase IV *in vitro*, there is little evidence that any of these phosphorylation events are required for NHEJ *in vivo* [58,59,62–64]. To date, the best candidate substrate for DNA-PK is DNA-PKcs itself. Sixteen *in vitro* autophosphorylation sites in DNA-PKcs have been identified [57,65–67] and it is likely that additional sites exist [20]. DNA-PKcs is also phosphorylated in response to DNA damage *in vivo*. Studies from our laboratories have shown that phosphorylation of serines 2612 and 2624 and threonines 2609, 2620, 2638, 2647 (which we have termed the ABCDE cluster [68]) as well as serine 2056 and threonine 3950 are all IR-inducible and DNA-PK-dependent *in vivo* [54,66,67,69] (Figure 3). Similarly, a proteomics study has reported that IR-induced phosphorylation of serine 2612 and threonines 2638 and 2647 occurs in cells in which the activity of the related protein kinase ATM (Ataxia-Telangiectasia Mutated) is inhibited, again, consistent with DNA-PK-dependent phosphorylation at these sites *in vivo* [70]. However, other studies have reported that ATM and the related PIKK, ATR (ATM-, Rad3-related), can phosphorylate serine 2612 and threonines 2609, 2638 and 2647 in response to IR or UV, respectively [71,72]. It is possible that all three PIKKs contribute to the phosphorylation of DNA-PKcs *in vivo*, depending on cell type, stage of cell cycle and/or the type or extent of DNA damage. Additional *in vivo* phosphorylation sites on serines 2671, 2674, 2675, 2677 [73] and 3205 [74] have been identified in proteomics screens (Figure 3), however, the kinases responsible and effects of phosphorylation at these sites on function is not known.

In vitro, autophosphorylation of DNA-PKcs results in loss of protein kinase activity and dissociation of phosphorylated DNA-PKcs from DNA-bound Ku (Figures 1E and F), suggesting that autophosphorylation of DNA-PKcs may serve to regulate the disassembly of the DNA-PK complex [75, 76]. Significantly, cells expressing DNA-PKcs in which serines and threonines in the ABCDE cluster have been mutated to alanine are more radiosensitive than cells expressing no DNA-PKcs at all [68]. The rate of the alternative DSB repair pathway, HDR, is also reduced in these cells [77]. Similarly, cells in which the protein kinase activity of DNA-PK is inhibited by a small molecule inhibitor are more radiosensitive than DNA-PKcs null cells and have reduced rates of HDR [78]. Moreover, although purified DNA-PKcs containing alanine mutations at the ABCDE phosphorylation sites is kinase active, it has reduced ability to dissociate from DNA-bound Ku *in vitro* [79, 80]. Together, these data support a model in which autophosphorylation of DNA-PKcs is required for its release from DSBs *in vivo* (Figures 1E and F). Recent studies using laser microbeam irradiation to induce DNA damage in cells expressing fluorescently tagged-DNA-PKcs also support this model, in that autophosphorylation-defective DNA-PKcs as well as kinase dead-DNA-PKcs were retained significantly longer at sites of laser-induced DNA damage than wt-DNA-PKcs [46]. It should also be noted however, that the effects of autophosphorylation on DNA-PKcs function may be highly complex *in vivo*, since phosphorylation at other regions of the molecule enhance the rate of HDR and phosphorylation at different sites can either positively or negatively affect DNA end-processing [81] (discussed in detail in [20]).

Processing of DNA ends

Once the DNA ends have been detected and secured, the next step in NHEJ is likely processing of the DNA termini to remove non-ligatable end groups and other lesions. IR-induced breaks are complex and may be highly variable from one DSB to another. Depending on the nature of the break, different processing enzymes may be required to remove blocking end groups, fill in gaps, and/or remove damaged DNA or secondary structure elements surrounding the break. Therefore, repair of different breaks may require different combinations of processing enzymes. Since NHEJ occurs in the absence of a DNA template or extended regions of microhomology, processing of the DNA ends has the potential to result in loss of nucleotides,

making NHEJ an inherently error prone process. Indeed, both *in vitro* assays and cell-based assays show that NHEJ proceeds with loss of sequence from DNA ends, and that this process is regulated at least in part by DNA-PKcs [68,81,82]. Candidate processing enzymes include Artemis, DNA polymerases μ (μ) and λ (λ), polynucleotide kinase (PNK), and possibly Aprataxin and PNK-like factor (APLF) and the Werner's syndrome helicase, WRN.

Artemis—Artemis possesses 5'-3' exonuclease activity and, in the presence of DNA-PKcs and ATP, acquires endonuclease activity towards DNA containing ds/ssDNA transitions as well as DNA hairpins [83,84]. Artemis can also remove 3'-phosphoglycolate groups from DNA ends *in vitro*, again consistent with a role in SSB or DSB repair [85]. Artemis is composed of an N-terminal metallo β -lactamase/ β -CASP nuclease domain [86] and a C-terminal region of uncertain function that is highly phosphorylated both *in vitro* and *in vivo* (discussed below) (Figure 4A). Inactivation of Artemis results in radiation-sensitive severe combined immunodeficiency (RS-SCID) in humans, and, similar to cells lacking DNA-PKcs, cells lacking Artemis accumulate unopened DNA hairpins at unprocessed coding joints during V (D)J recombination [87,88]. Moreover, Artemis interacts with DNA-PKcs providing a mechanism whereby it may be recruited to the DSB [83,89] (Figure 1(G)). However, although Artemis-deficient cells are radiation sensitive they do not have major defect in DSB repair, suggesting that Artemis is required for the repair of only a subset of DNA damage events *in vivo* [90,91].

Both DNA-PKcs and ATM phosphorylate Artemis *in vitro* [62,65] and Artemis is highly phosphorylated at both basal and DNA damage-induced sites *in vivo* [62,89–94] (Figure 4A). It has been suggested that phosphorylation of Artemis by DNA-PKcs is required for Artemis' endonuclease activity [83,95]. However, mutation of DNA-PKcs/ATM phosphorylation sites in Artemis had no effect on its endonuclease activity *in vitro* [62]. Instead, we and others have proposed that autophosphorylation of DNA-PKcs may be required for the endonuclease activity of Artemis, by facilitating access of Artemis to its DNA substrates [62,96].

The role of phosphorylation on Artemis function is far from clear. It has been suggested that DNA-PK kinase activity is required for recruitment of Artemis to damaged DNA [97], whereas other studies suggest that DNA damage-induced phosphorylation of Artemis *in vivo* is largely ATM-dependent [90–93,98,99] (although DNA-PKcs may phosphorylate Artemis at high doses of IR and/or in ATM-deficient cells [91,94,99]). Moreover, Artemis has also been shown to function in an ATM-dependent pathway for the repair of a subset of complex DNA lesions *in vivo* [100] and ATM-dependent phosphorylation of Artemis has been linked to recovery from the IR-induced G2/M checkpoint [94]. Thus, it seems likely that Artemis is involved in multiple aspects of the DNA damage response and that its activity may be regulated by ATM and/or DNA-PK.

DNA polymerases μ and λ —Processing of complex, IR-induced DNA damage can lead to the creation of DNA gaps that require the action of DNA polymerases for their repair. Members of the DNA pol X family of DNA polymerases, pol μ , pol λ and terminal deoxyribonucleotidyltransferase (TdT), have all been implicated in NHEJ. TdT interacts with Ku but is only expressed in lymphocytes and so its function is likely limited to V(D)J recombination (reviewed in [101]). In contrast, pol μ and pol λ are widely expressed and are thought to have more widespread roles in NHEJ (reviewed in [101]). DNA pol μ and DNA pol λ each contain an N-terminal BRCT (BRCA C-terminal) domain that is required for their functions in NHEJ (Figures 4B and C). DNA pol μ and λ are recruited to DSBs via their interactions with Ku and the X4-L4 complex (reviewed in [102]) (Figure 1(K)). Which polymerase is recruited to the DSB may depend on the type of damage to be repaired. While pol μ and pol λ carry out similar gap-filling reactions, they differ in their requirement for a DNA template. Pol λ is largely template-dependent [103,104], whereas pol μ is less template

dependent [104,105] and has the unique ability to direct template-independent synthesis across a DSB with no terminal microhomology [106]. Precisely when pol μ and pol λ are recruited to the DSB and whether their recruitment requires Ku or X4-L4 *in vivo* remains to be determined. Since cells lacking one or both polymerases are not highly sensitive to IR [107], it seems likely that pol μ and pol λ are only required for the repair of a small subset of DNA breaks (reviewed in [105]). DNA pol λ is phosphorylated in response to IR [70], but the kinase(s) responsible and the effects on function are not known.

Polynucleotide kinase (PNK)—PNK has both 3'-DNA phosphatase and 5'-DNA kinase activities, and thus is ideally suited to remove non-ligatable end groups from DNA termini [108]. Indeed, several studies have pointed to a role for PNK in NHEJ. First, the N-terminal forkhead associated (FHA) domain of PNK (Figure 4D) interacts with casein kinase 2 (CK2)-phosphorylated XRCC4, providing a potential mechanism to recruit PNK to the DSB [109] (Figures 1I and J). *In vitro* DSB end-joining studies also indicate a role for PNK in NHEJ [110]. Moreover, knockdown of PNK in human cells renders them radiosensitive and defective in DSB repair [111]. Radiation sensitivity was attributed to a defect in NHEJ since PNK knockdown cells were proficient at sister chromatid exchange but epistatic with the DNA-PKcs-defective cell line, M059J [112]. PNK is also phosphorylated *in vivo* in response to IR [70], however, the kinase(s) responsible and the effects on function are not known.

Aprataxin and PNK like factor (APLF)—A potential new player in NHEJ is APLF [113–115] (also called C2orf13, Xip1 [116], and PALF [117]). APLF has both endonuclease and exonuclease activities, consistent with a role in processing DNA ends [117]. APLF contains a PNK-like FHA domain, and, like PNK, interacts with CK2-phosphorylated XRCC4 [113, 114, 116, 117] (Figure 4E). APLF also interacts with Ku and down-regulation of APLF causes defects in DSB repair [113, 114, 117]. Together, these data support a possible role for APLF in NHEJ (Figure 1(L)). APLF is phosphorylated in an ATM-dependent manner in response to DNA damage but the function of phosphorylation is not known [114, 116].

The Werner's syndrome protein (WRN)—WRN is a member of the RecQ helicase family that possesses DNA-dependent ATPase, 3'-5' DNA helicase, strand annealing and 3'-5' exonuclease activities. Inactivation of WRN is associated with premature aging, cancer predisposition and genomic instability (reviewed in [118]). WRN interacts with Ku which stimulates its exonuclease activity [119–121]. WRN is phosphorylated *in vitro* by DNA-PK and is phosphorylated in a DNA-PK-dependent manner in cells [122]. WRN also interacts with the X4-L4 complex which also stimulates its exonuclease activity *in vitro* [123]. Thus, although WRN negative cells are not highly radiation sensitive [118], several lines of evidence support a role in NHEJ (Figure 1(L)).

Other potential processing enzymes—It is possible that other processing enzymes may also play a role in NHEJ. One candidate, tyrosyl-DNA phosphodiesterase 1 (Tdp1) removes 3'-phosphoglycolate groups from DNA ends [124], however, recent studies suggest that Tdp1 is primarily involved in the repair of SSBs not DSBs [125]. Another possible processing enzyme is the Mre11 nuclease, however, although Mre11 is required for NHEJ in *S. cerevisiae* [126], it is not thought to play a role in vertebrate NHEJ [127].

Logistically, end-processing must occur prior to ligation of the DNA ends, however, precisely when the processing enzymes are recruited to the DSB is not clear. It is possible that many aspects of DNA end-processing occur within a multi-protein complex composed of Ku, XRCC4, DNA ligase IV, and possibly DNA-PKcs, that is assembled at the DSB (Figure 1). It is also possible that the specific enzymes involved in end-processing and their order of recruitment to the DSB may be quite flexible, depending on the nature of the break and other factors [128, 129].

Ligation of the DNA ends

The XRCC4-DNA ligase IV complex (X4-L4)—Once the DNA ends have been processed they must be ligated to repair the DNA. Ligation is carried out by DNA ligase IV, which exists in complex with XRCC4 (referred to here as X4-L4) (Figure 1(J)).

XRCC4 is required for both NHEJ and V(D)J recombination [130,131]. It is composed of a globular head domain, an elongated α -helical stalk and a C-terminal region of unknown function [132] (Figure 5A). XRCC4 has no known enzymatic activity but rather acts as a scaffolding protein, facilitating the recruitment of other NHEJ proteins to the break. XRCC4 itself is a homodimer and two dimers can interact to form tetramers [132,133]. The most well characterized binding partner of XRCC4 is DNA ligase IV. DNA ligase IV contains two C-terminal BRCT domains separated by a linker region that interacts with the α -helical region of XRCC4 to form a highly stable complex [134,135] (Figure 5B). XRCC4 stabilizes DNA ligase IV and stimulates its activity [136,137]. Interestingly, DNA ligase IV has the unusual property of being able to ligate one DNA strand at a time [138], perhaps allowing processing enzymes to act simultaneously on end groups on the opposite strand.

Consistent with its role as a scaffolding protein, XRCC4 and/or the X4-L4 complex interacts with Ku [36,38,39,139], PNK [109], APLF [113,114] and XLF [140–142] as well as with DNA [143]. However, precisely when the X4-L4 complex (and presumably its associated factors) is recruited to the DSB is not clear. NHEJ has been assumed to proceed in a stepwise fashion with binding of Ku and DNA-PKcs, followed by recruitment of the X4-L4 complex [26,37]. Indeed, biochemical studies suggest that DNA-PKcs is required for recruitment of the X4-L4 complex to chromatin after damage [144]. However, recent laser microbeam irradiation experiments have suggested that although recruitment of XRCC4 to sites of damage requires Ku, it does not require DNA-PKcs [36,44] (although localization at the break may be stabilized by the presence DNA-PKcs [44]). Thus, it is possible that DNA-PKcs and X4-L4 may be recruited to the DSB independently, rather than in a sequential manner as was originally supposed.

XRCC4 is highly phosphorylated *in vivo* [130] and its phosphorylation, as detected by a mobility shift on SDS PAGE, is enhanced by DNA damage [144,145]. It has been suggested that DNA-PK is required for DNA damage-induced phosphorylation of XRCC4 [144,145] and that DNA-PKcs promotes ligation by X4-L4 [146]. Indeed, XRCC4 is phosphorylated by DNA-PK *in vitro* [130,133] (Figure 5A). However, DNA-PK phosphorylation sites in XRCC4 are not required for either NHEJ or V(D)J recombination [58,147] and the role for DNA-PK-mediated phosphorylation in the function of XRCC4 still remains unclear. As discussed above, XRCC4 is also phosphorylated by CK2 [109] which creates a binding site for the FHA domain of PNK [109] and APLF [113,114,116,117], facilitating their recruitment to the DSB. XRCC4 is also SUMO-ylated *in vivo*, and this modification is important for nuclear localization of XRCC4 and DSB repair [148].

XLF—XLF is similar in structure to XRCC4 [140,149,150] (Figure 5C), interacts with XRCC4 *in vitro* [140,149] and is required for NHEJ and V(D)J recombination [140–142]. *In vitro*, XLF stimulates the activity of DNA ligase IV towards non-compatible DNA ends, suggesting that XLF may only regulate the activity of X4-L4 under a subset of conditions [138,149,151–154]. Like XRCC4, XLF also interacts with DNA. This interaction is highly dependent on the length of the DNA molecule and is enhanced by Ku [40,151]. Surprisingly, given the ability of XLF to interact with XRCC4, XRCC4 was not required for the recruitment of XLF to sites of DNA damage *in vivo* [40]. However, the presence of XRCC4 did result in XLF being retained longer at the damage sites, suggesting that XLF is recruited to the DSB through interaction with DNA-bound Ku, but stabilized at the break by interaction with X4-L4. Like XRCC4, XLF is phosphorylated *in vitro* at C-terminal sites by DNA-PK (Figure 5C) and is phosphorylated

by both ATM and DNA-PK *in vivo*, however, phosphorylation is not required for NHEJ and its function remains unclear [59].

CONCLUSIONS

In conclusion, NHEJ has emerged as one of the major pathways for the repair of IR-induced DSBs in mammalian cells. Many of the proteins required for NHEJ have been identified and characterized at a biochemical and/or cellular level and, in many cases, animal models have been generated [19]. However, although many protein-protein and protein-DNA interactions have been identified at the biochemical level, until recently how the NHEJ proteins interact in the cell has been largely unknown. The emerging challenge is to understand the choreography and dynamics of recruitment and release of each of the NHEJ factors to the break *in vivo*.

As discussed above, the use of laser microbeam irradiation to induce DNA damage in living cells has provided intriguing new insights into the interplay between the various components of the NHEJ reaction. However, it should be noted that laser microbeam irradiation can introduce large, perhaps non-physiological, amounts of damage within the nucleus (discussed in [35,46,155]). Indeed, some studies have reported that DNA-PKcs and Ku only localize to laser-induced sites of DNA damage when high power lasers are used [156]. Other approaches, such as the introduction of defined DSBs in the genome followed by chromatin immunoprecipitation (ChIP) analysis are also beginning to yield new information on the kinetics of DSB repair in the cell and the proximity of various proteins to the break [157, 158] and are likely to be important tools as researchers tease apart the kinetics of the various DSB repair pathways. However, these methods also have drawbacks. For example, DSBs created by endonucleases are not formed instantaneously and the DNA ends at these breaks are complementary and the bases unmodified, unlike IR-induced DSBs, which are often not directly ligatable, complex and created much more rapidly. Furthermore, in yeast, IR-induced and endonuclease-induced DSBs are differentially processed in a cell cycle-dependent manner [159].

The rapid pace of discovery in this field means that new models for NHEJ continue to be developed, and that new questions are raised. As discussed above, recent studies confirm that Ku plays a central role not only in detection of the DSB but also in the recruitment and or stabilization of other NHEJ proteins at the break. However, some experiments have questioned whether recruitment of DNA-PKcs is required for recruitment of the X4-L4 complex to the DSB and it is unclear whether DNA-PKcs is required only for initiation of end-processing (Figure 1(F)), or whether the holoenzyme remains assembled at the DSB until repair is complete (Figure 1(M)). In addition, a picture has emerged in which end-processing (trimming, fill-in and ligation of each separate strand) may occur in a flexible manner, thus different proteins may be recruited depending on the nature of the break. One intriguing and still unanswered question in NHEJ is how Ku is released from the DNA prior to ligation. The structure of the core DNA binding domain of Ku70/80 suggests that either Ku must back off the DNA prior to ligation or that it is removed from the DNA by proteolysis. A recent study has shown that Ku80 is modified by ubiquitylation *in vitro*, and that this has the potential to regulate the release of Ku from DNA, at least in cell extracts [160] (Figure 1(N)).

While it is clear that cells which lack DNA-PKcs or in which DNA-PK activity has been inhibited are highly radiosensitive and have defects in DSB repair, the precise role of DNA-PKcs in NHEJ is still not fully understood. Studies from several laboratories, including our own, have shown that the major role of DNA-PKcs in NHEJ appears to be autophosphorylation-dependent regulation of access to the DNA ends [20,21,62]. Therefore, DNA-PKcs likely plays a regulatory role in NHEJ. This is consistent with the fact that unlike Ku, XRCC4 and DNA ligase IV, DNA-PKcs is not conserved in evolution and seems only to be required for NHEJ

in higher eukaryotes (discussed in [161]). In the absence of a high-resolution structure for DNA-PKcs or the DNA-PKcs-Ku-DNA complex, precisely how DNA-PKcs interacts with Ku and DNA is still unclear. Low-resolution structures suggest that DNA-PKcs binds dsDNA via a central cavity consistent with a role for DNA-PKcs in protection of DNA ends [162]. Moreover, a low-resolution structure of the DNA-PKcs-Ku-DNA complex is consistent with interaction of DNA-PKcs and Ku with DNA ends to form a synaptic complex as suggested in Figure 1(E) [163,164]. We speculate that after assembly of the complex, autophosphorylation of DNA-PKcs induces a conformational change that releases DNA-PKcs from the Ku-DNA complex, thus making the DNA ends accessible for downstream processing enzymes.

It has also become clear that DNA-PKcs does not have a major role in phosphorylating other components of the NHEJ pathway and/or that phosphorylation of NHEJ factors by DNA-PK is not required for NHEJ. This leads to the question of whether the main substrate of DNA-PKcs is itself, or whether, like ATM, it does indeed phosphorylate multiple substrates *in vivo* [165–167]. If the later, then it seems likely that additional physiological substrates of DNA-PK might be found outside the canonical NHEJ pathway. Indeed, like ATM and ATR, DNA-PK contributes to the DNA damage-induced phosphorylation of histone H2AX on serine 139 [168]. This phosphorylated form of H2AX, termed γ -H2AX, is widely regarded as a marker for unrepaired DSBs, and clusters of γ -H2AX molecules, termed foci, serve to recruit and/or retain other DSB repair proteins at the sites of DNA damage [169]. Another recently identified substrate of DNA-PKcs is the pro-survival protein kinase PKB/Akt. DNA-PKcs was shown to interact with PKB/Akt and was required for DNA damage induced phosphorylation and activation of PKB/Akt [170]. It is possible that proteomics approaches may yield additional DNA-PK substrates. A recent proteomics screen in which antibodies to phosphorylated SQ/TQ sites were used to immunoprecipitate proteins from irradiated cells identified over 900 IR-induced phosphorylation sites in over 600 protein substrates [70]. Although this study attributed phosphorylation to ATM and/or ATR, given that DNA-PK also phosphorylates SQ/TQ sites, some of these sites could equally represent DNA-PK-dependent phosphorylation events. Regardless of what its physiological substrates are, the ability of small molecule inhibitors of DNA-PKcs to radiosensitize cells suggests that DNA-PK may be an attractive therapeutic target as a radiation sensitizer (reviewed in [60,171,172]).

As discussed above, IR-induced DNA damage is highly complex, and produces damage to bases and production of SSBs as well as DSBs. Indeed, the number of damaged bases and SSBs far outweighs the number of DSBs produced by IR [6]. It is therefore likely that BER and SSB repair pathways must function in close proximity to DSB repair pathways. Interestingly, several potential connections between the proteins involved in BER, SSB repair and NHEJ are beginning to emerge. For example, PNK and APLF interact not only with XRCC4 [109,113,114,116,117] but also with XRCC1, a protein required for both BER and SSB repair [113,173,174]. It is also interesting to note that XRCC1 has been identified as an IR-inducible target of DNA-PK [175]. Proteins involved in BER and SSB repair have also been implicated in alternative DSB repair pathways [13–16]. Thus, it seems likely that NHEJ, BER and SSB repair pathways function in a coordinated manner to repair IR-induced DNA damage.

Another outstanding question in the field is how a cell decides to repair DSBs via NHEJ, HDR, or alternative end-joining pathways. This area is currently the topic of intense study [2,11,176,177]. One potential mechanism appears to be via cell cycle regulated expression of the CtIP/Sae2 protein, which stimulates resection at the DSB by the MRN complex, thus promoting HDR in S and G2 phases of the cell cycle (reviewed in [178]). Another critical, yet unresolved, question regarding pathway choice is what regulates recognition of DSBs by Ku versus the MRN complex, which is required not only for initiation of HDR, but also for activation of ATM-dependent signaling pathways [165–167]. Finally, we note that ATM phosphorylates several NHEJ proteins, including Artemis [94] and XLF [59] as well as DNA-PKcs [71,179],

suggesting that the PIKK family members act together to coordinate the DNA damage response rather than in separate pathways. Understanding how these multiple pathways are connected and regulated both temporally and spatially will provide critical insights into the mechanisms by which cells deal with the deleterious effects of DNA damage and prevent genomic instability.

Abbreviations

Aa	amino acid
APLF	<u>A</u> prataxin- and <u>P</u> NK- <u>L</u> ike <u>F</u> actor
ATM	<u>A</u> taxia- <u>T</u> elangiectasia <u>M</u> utated
ATR	<u>A</u> TM-, <u>R</u> ad3-related
BER	base excision repair
DNA-PK	DNA-dependent protein kinase (composed of DNA-PKcs plus Ku assembled at a DSB)
DNA-PKcs	catalytic subunit of the DNA-dependent protein kinase
ds	double-stranded
DSB	DNA double strand break
HDR	homology directed repair
IR	ionizing radiation
kd	kinase dead
NHEJ	non-homologous end-joining
PIKK	phosphatidylinositol 3-kinase-like protein kinase
PNK	polynucleotide kinase
SCID	severe combined immunodeficiency
SSB	DNA single strand break
wt	wild type
X4-L4	the XRCC4-DNA ligase IV complex
XLF	<u>X</u> RCC4- <u>L</u> ike- <u>F</u> actor (also known as Cernunnos)

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Figure 1. A model for NHEJ

(A) IR induces multiple forms of DNA damage including DSBs that contain non-ligatable end groups such as 3'-phosphate and 3'-phosphoglycolate groups (indicated by $\blacklozenge$). (B) The Ku heterodimer (orange) binds the ends of the DSB, tethering the ends together. Recruitment of Ku to the DSB occurs independently of other known NHEJ or DSB repair proteins, consistent with Ku acting as the cornerstone of the NHEJ pathway. (C) Ku translocates inwards, allowing recruitment of DNA-PKcs (blue) such that it binds the extreme termini of the break (D). Recruitment of DNA-PKcs to the DSB requires Ku but no other NHEJ or DSB repair factors. Two DNA-PK molecules (DNA-PKcs bound to DNA-bound Ku) interact to tether the DSB together in what has been termed a "synaptic complex". This triggers autophosphorylation (yellow circles) of DNA-PKcs in *trans* (E), inducing a conformational change that causes release of the DNA ends and/or release of phosphorylated DNA-PKcs from the complex. Whether DNA-PKcs is released prior to recruitment of the X4-L4 complex (green) and its associated factors (F), or whether it remains part of a multi-protein complex until repair is completed (M) is not known. Inhibition of the protein kinase activity of DNA-PKcs (step E), prevents dissociation of DNA-PKcs (step F), blocking access of NHEJ or other repair factors to the DSB, resulting in radiation sensitivity. (G) A portion of the total cellular DNA-PKcs interacts with the nuclease Artemis (red), but if or when Artemis is released from the DNA-PKcs complex (H) is not known. (I) PNK (pink) interacts with XRCC4 suggesting that it is recruited to the break with the X4-L4 complex (green) (J). XLF (yellow) and DNA pol μ and λ (purple) interact with both X4-L4 and Ku, suggesting that they are recruited after or at the same time as X4-L4 is recruited to the Ku-DNA complex (K). Other processing enzymes such as WRN and APLF (shown in grey) may also be recruited through interactions with DNA-bound Ku, XRCC4 and/or the X4-L4 complex (L). The order of recruitment of processing factors may be flexible and depend on the precise type of DNA damage present at the DSB. Multiple protein-protein and protein-DNA interactions may stabilize the formation of the complex at the DSB as well as aid in retention of NHEJ factors at the break. Once the ends are processed, the X4-L4 complex ligates the ends, repairing the break. Ligation of incompatible DNA ends is aided by the regulatory factor, XLF. How the various factors are released after repair is unknown, however, it is possible that ubiquitylation and/or proteolysis may be involved (N). Reactions requiring or enhanced by the presence of DNA-bound Ku are shown in red.

Figure 2. Major features of Ku70 and Ku80 polypeptides

Domain boundaries, phosphorylation sites (red), protein-protein interaction sites and interacting proteins (yellow ovals) are shown for **(A)** Ku70 and **(B)** Ku80. Domain boundaries for the von Willebrand domain (vWa) (amino acids (aa) 35–249), Ku core (aa 266–529) and SAP domains (aa 573–607) of Ku70 and the vWa (aa 7–237), Ku core (aa 244–543) and C-terminal domains (aa 590–709) of Ku80 were obtained from the NCBI database. The location of putative nuclear localization sequences (NLS) in Ku70 (aa 539–556) and Ku80 (aa 561–569) are from [180,181]. *In vitro* DNA-PK phosphorylation sites in Ku70 (serine 6) and Ku80 (serines 577 and 580 and threonine 715) are indicated in red [63]. Amino acids 720–732 of Ku80 contain the DNA-PKcs binding region [29,30].

Figure 3. Major features of DNA-PKcs

Domain boundaries and major features are represented as in Figure 2. The N-terminal domain extends from aa 1–2908, the FAT domain is from aa 2908–3539; the PIKK domain from aa 3645–4029 and the FATC domain from 4096–4128. *In vivo* phosphorylation sites between threonine 2609 and threonine 2647 (termed the ABCDE cluster) are from [21,68]. *In vivo* phosphorylation sites between serine 2023 and serine 2056 (the PQR cluster) are from [81]. The 2671 cluster, which contains four sites between threonine 2671 and 2677 is from [73]. *In vivo* phosphorylation of threonine 3950 and serine 3205 were describe in [80] and [57] respectively. Reported interaction sites for Ku are from reference [50] (amino acids 3002–3850) and the putative PIKK regulatory domain (PRD) is from [51].

Figure 4. Major features of the processing enzymes Artemis, PNK, APLF and pol X family members μ and λ .

Domain boundaries and major features of Artemis, PNK, APLF, pol μ and pol λ are represented as in Figure 2. **(A)** Artemis: The metallo β lactamase domain (aa 1–155) and β -CASP domain (aa 156–385) are as described in [86]. Amino acids 398–403 are required for interaction with DNA-PKcs [89,95]. The C-terminal region of Artemis is highly phosphorylated at multiple site both *in vitro* and *in vivo* [62,65,89,90,92,93] but the effects of phosphorylation on function are not known. **(B and C)** Pol μ and λ : Domain boundaries for the lyase (aa. 156–227) and polymerase (aa 227–494) domains of pol μ are based on a structure based alignment of pol X family members [103,182]. The BRCT (aa. 29–109) domain is as described in the NCBI

database. The lyase (aa 242–327), polymerase (aa 327–575) and the BRCT domains (aa 35–125) of pol lambda are from [103,182]. **(D)** PNK: Domain boundaries for the FHA (aa 6–110), phosphatase (aa 145–337) and kinase (aa 341–521) domains are based on the X-ray crystal structure [183]. CK2 phosphorylated XRCC4 and XRCC1 interact with the FHA domain [109,174]. Serines 114 and 126 are IR-induced phosphorylated sites of unknown function [70]. **(E)** APLF: Amino acids 20–102 compose the FHA domain. XRCC1/XRCC4 bind within the FHA domain [113,114]. The poly(ADP-ribose) binding zinc finger (PBZ) regions (aa 377–398 and aa 419–440) of APLF are shown in purple [115]. Although APLF has endonuclease and exonuclease activity, these domains have yet to be defined.

A

XRCC4

B

DNA ligase IV

C

XLF

Figure 5. Major features of DNA ligase IV, XRCC4 and XLF

Domain boundaries and major features of DNA ligase IV, XRCC4 and XLF are represented as in Figure 2. **(A)** XRCC4: The head (aa 1–115) and stalk domains (aa 135–233) of XRCC4 are based on the x-ray crystal structure [184]. The region of XRCC4 required for dimerization (aa 119–155) is from [132]; DNA ligase IV interacts with XRCC4 between amino acids 173–195 [134]; CK-2 phosphorylates XRCC4 at threonine 233 [109] and DNA-PK phosphorylates XRCC4 at serine 260 and serine 318 *in vitro* [58]. The XLF binding site (aa 63–99) is from [150]. **(B)** DNA ligase IV: The N-terminal (aa. 14–203) and ligase domain (aa. 248–451) boundaries are as found in the NCBI database. The XRCC4 binding site in DNA ligase IV (aa. 755–782; [134]) is located between the BRCT domains (aa. 661–731 and aa. 829–898); *In vitro*, DNA ligase IV is phosphorylated at threonine 650, serine 668 and serine 672 [64]. **(C)** XLF: The head (aa 1–135) and stalk (aa 135–233) domains are based on the X-ray crystal structure [184]. Leucine 115 is crucial for XRCC4 binding and amino acids 125–224 are involved in homodimerization [150,184]. *In vivo*, XLF is phosphorylated at serine 245 by DNA-PK and serine 251 by ATM but the effect of phosphorylation on function is unknown [59].