24

DNA Double-Strand Break Repair Through Non-Homologous End-Joining: Recruitment and Assembly of the Players

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1. Introduction

DNA, this vitally important genetic macromolecule, is under constant assault via endogenous and exogenous agents which cause damage to DNA and thus to cells leading to genomic instability. The primary endogenous cause of DNA damage is caused during continuous replication of DNA at the S phase of the cell cycle effecting spontaneous mutations. Other endogenous DNA damaging agents are reactive oxygen species (ROS) produced as metabolic byproducts. Additionally, breaks are introduced to DNA in the process of recombination, *e.g.*, V(D)J recombination in immune systems and meiotic recombination in reproductive organs. The exogenous DNA damaging agents are ionizing radiations and chemical compounds, which are intercalated into major or minor grooves of DNA strand or form chemical bond with bases.

DNA damages include base elimination, modification, cross-linking and strand break. Strand break includes single-strand break (SSB) and double-strand break (DSB). Among these various types of DNA damages, DSB is considered most fatal. Hence healing DSB is vital to circumvent genomic instability encompassing chromosomal aberrations, translocations and tumorigenesis. Eukaryotes have evolved two major pathways to repair DSBs, *i.e.*, homologous recombination (HR) and non-homologous end-joining (NHEJ). This chapter will review the mechanisms of the latter, especially how the players are recruited to the sites of DSBs and are assembled into multi-protein repair machinery.

2. DNA double-strand break repair through non-homologous end-joining pathway

2.1 Homologous Recombination and Non-Homologous End-Joining

HR is a reaction wherein the genetic material is exchanged between two similar or identical strands of DNA. In the repair of DSB through HR, undamaged DNA serves as a template to reconsitute the original sequence across the break. On the other hand, NHEJ is the direct rejoining of the broken DNA ends without much regard for homology at these ends.

Therefore, NHEJ may sometimes incur nucleotide deletions or insertions at the junction or joining with incorrect partner, leading to chromosomal abberations like duplications, inversions or translocations. Hence it is considered that NHEJ is less accurate than HR but, nevertheless, important especially in vertebrates.

In HR, the template should be found in homologous chromosome or in sister chromatid. Organisms like budding yeast can avail homologous chromosome as the template. However, vertebrate can utilize only sister chromatid, but not homologous chromosome, as the template for HR and, therefore, the repair of DSB through HR is limited to late S and G2 phases. The majolity of the cells reside in G0 or G1 phases in vertebrate body, where only NHEJ can operate.

Additionally, only small portions of the genome in vertebrate are encoding protein or functional RNA and other portions are intervening or repetitive sequences. These regions may have important roles in the structural maintenance of the genome, proper replication/segregation of the genome or spatiotemporal regulation of the genen expression. Nevertheless, small deletion or insertion of nucleotides might be tolerated in most portion of the vertebrate genome.

Finally, whereas HR is utilized in meiotic recombination in reproductive organs, NHEJ is utilized in V(D)J recombination in immune system to establish diversity of immunogloblins and T cell recepters. Thus, genetic defect in either one of NHEJ components results not only in elevated sensitivity toward radiation and radiomimetic agents but also in immunodeficiency.

2.2 Processes of NHEJ

NHEJ process may be divided into three steps, i.e., (i) detection, (ii) processing and (iii) ligation of DSB ends (Fig.1). The detection and ligation steps comprises the core reaction while the processing step is required only when the ends are not readily ligatable. In the detection step, Ku protein, heterodimer consisting of Ku70 and Ku86 (also known as Ku80), first binds to the ends of double-stranded DNA and then recruits DNA-PK catalytic subunit (DNA-PKcs). The complex consisting of Ku70, Ku86 and DNA-PKcs is termed DNA-dependent protein kinase (DNA-PK). Upon binding of DNA-PKcs to DNA ends, it exerts kinase catalytic activity to phosphorylate substrate proteins. Thus, DNA-PK is considered the molecular sensor of DSB, triggering the signalling cascade. At the final ligation step, DNA ligase IV in a tight association with XRCC4 catalyzes the reaction to join the two DNA ends. XRCC4-like factor, XLF, which is also known as Cernunnos, is also essential at this step, especially when two ends are not compatible. Thus, six polypeptides, i.e., Ku70, Ku86, DNA-PKcs, DNA ligase IV, XRCC4 and XLF are core components of NHEJ. Processing step might involve a number of enzymes depending on the shape of each DNA end and compatibility of two ends to be ligated. Presumed processing enzymes contain Artemis, DNA polymerase μ/λ, polynucleotide kinase/phosphatase (PNKP), Aprataxin (APTX) and Aprataxin and PNKP-like factor (APLF, also known as PALF, C2orf13 or Xip1).

2.3 Components of NHEJ

2.3.1 Ku

Ku protein was initially found as the antigen of autoantibody in a patient of polymyositis-scleroderma overlap syndrome (Mimori et al., 1981). Biochemical approach, including immunoprecipitation of [^{32}P]orthophosphate or [^{35}S]methionine-labeled cell extract and

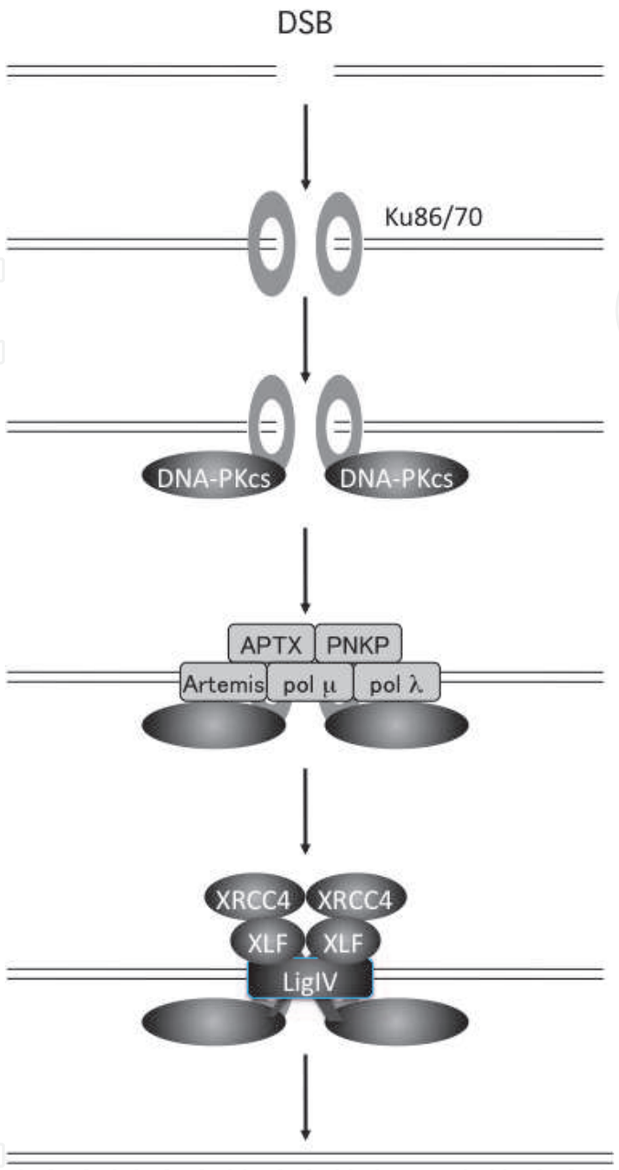

Fig. 1. Repair of DSB through NHEJ.

immunoaffinity purification, lead to identify Ku as a DNA-binding protein made up of two subunits of 70,000Da and 80,000Da, respectively, which are now known as Ku70 and Ku80 (or Ku86) (Mimori et al., 1986). It is also estimated that Ku is an abundant protein, existing as 400,000 copies in logarithmically growing HeLa cells. Protein-DNA interaction studies, including footprint analysis, led to the finding that Ku binds to the ends of double-stranded DNA without requirement for specific sequence (Mimori & Hardin, 1986). Because of this striking property, possible role of Ku in DNA repair or in transposition was suspected. In early 1990s, Ku was found to be an essential component of DNA-PK (Dvir et al., 1992, 1993; Gottlieb and Jackson, 1993). It was also found that Ku80 is equivalent to XRCC5 (X-ray repair cross complementing) gene product, which is missing in X-ray sensitive rodent cell lines including *xrs*-5, -6, XR-V9B and XR-V15B (Taccioli et al., 1994; Smider et al., 1994).

These cell lines also exhibit defect in V(D)J recombination, indicating the role of Ku in this process. Ku80 knockout mice showed immunodeficiency and radiosensitivity, like *scid* mice (below) and also exhibited growth defect; body weight was 40-60% of age-matched control (Nussenzweig et al., 1996; Zhu et al., 1996). Ku70 knockout mice also showed immunodeficiency, radiosensitivity and growth defect. However, immunological defect in Ku70 knockout mice was less severe than in Ku80 knockout mice, as it shows partial production and differentiation of T cells (Gu et al., 1997; Ouyang et al., 1997). Ku is also implied to play critical roles in telomere capping in mammalian cells (Hsu et al., 2000).

Homologues of Ku proteins were identified not only in mammals but also in other eukaryota including budding yeast, where it is referred to as HDF1 and HDF2 (high- affinity DNA binding factor), or Yku70 and Yku80, respectively (Feldmann & Winnacker, 1993; Milne et al., 1996; Feldmann et al., 1996; Boulton & Jackson, 1996). Yeast Ku is shown to play important roles in NHEJ, telomere maintenance and silencing (Boulton & Jackson, 1996, 1998; Porter et al., 1996).

Ku70 and Ku80 show low but significant sequence similarity, indicating common evolutional origin, and share a similar structural configuration (Dynan & Yoo, 1998; Gell & Jackson, 1999). Expectedly, "single" Ku orthologue was identified in bacteria and in bacteriophage (Weller et al., 2002; d'Adda di Fagagna et al., 2003). As revealed by X-ray crystallography, Ku 70 and Ku80 fold to form an asymmetric ring shaped structure forming an aperture large enough to let DNA thread through it; thus playing a crucial role in DSB recognition (Walker et al., 2001). The core of Ku required to form dimer and aperture is conserved among all Ku orthologues. Both of Ku70 and Ku80 bear von Willebrant factor A domain, which may be essential for heterodimer formation. The C-termial of Ku70 bears SAP domain, which may mediate DNA binding, and the C-terminal of Ku80 bears a conserved motif to interact with DNA-PKcs (Gell & Jackson, 1999; Falck et al.., 2005).

Ku translocates along DNA in an ATP-independent manner, allowing several dimers to bind on a single DNA molecule (Zhang and Yaneva, 1992; Bliss and Lane, 1997). Ku was identified also as a ssDNA dependent ATPase stimulating the DNA polymerase α primase activity (Vishwanatha and Baril, 1990; Cao et al., 1994) and as an ATP dependent DNA helicase II (HDH II) (Tuteja et al., 1994). Recent study demonstrated that Ku has 5'-RP/AP lyase activity, nicking 3'-side of abasic site (Roberts et al., 2010). Thus, Ku might exert multiple functions, not only binding to DSBs but also activating damage signal via DNA-PKcs and processing DSB ends removing the obstacle for ligation.

2.3.2 DNA-PKcs

DNA-PK activity was first found as an activity to phosphorylate Hsp90 in the presence of double-stranded DNA in the extracts of HeLa cell, rabbit reticulocyte, Xenopus egg and sea urchin egg (Walker et al., 1985). DNA-PK was purified from Hela cell nuclei as a 300-350 kDa protein, which is now called DNA-PKcs for DNA-PK catalytic subunit (Carter et al., 1990; Lees-Miller et al., 1990). Later it was found that Ku is an essential component of DNA-PK and that DNA-PK requires binding to DNA ends to be activated (Dvir et al., 1992, 1993; Gottlieb and Jackson, 1993). Following the finding that XRCC5 is equivalent to Ku80, DNA-PKcs is found to correspond to XRCC7, which is deficient in *scid* (severe combined immunodeficiency) mouse (Kirchgessner et al., 1995; Blunt et al., 1995; Peterson et al., 1995), lacking mature B and T cells due to a defect in V(D)J recombination (Bosma et al., 1983; Lieber et al., 1988; Fulop & Phillips, 1990; Biederman et al., 1991). Scid due to defect in DNA-

PKcs is also found in horse (Wiler et al., 1995; Shin and Meek, 1997) and in dog (Meek et al., 2001). M059J, a human glioma cell line, defective in DNA-PKcs, also showed radiosensitivity with defective DSB repair (Lees-Miller et al., 1995). Recently, DNA-PKcs missense mutation was identified in human radiosensitive T- B- severe combined immunodeficiency (TB-SCID) (van der Burg et al., 2009). Cells from the patient exhibit normal DNA-PK activity but may have defect in Artemis activation (below).

Cloning of gene revealed that DNA-PKcs is a 4,127 amino acid polypeptide, one of the largest molecules in the cell (Hartley et al., 1995). The carboxy-terminal between amino acid residues 3719 – 4127 compose the catalytic domain that is categolized into phosphatidylinositol-3 kinase and like kinase (PIKK) family (Hartley et al., 1995; Poltoratsky et al., 1995). PIKK family include ataxia-telangiectasia mutated (ATM) (Savitsky et al., 1995) and ATM- and Rad3-related (ATR) (Cimprich et al., 1996), both of which are protein kinases with roles in DNA repair and cell cycle checkpoint as sensors of DNA damages. Although orthologues of ATM and ATR can be found in fruit fly, nematoda, plants and yeast, DNA-PKcs has been found only in vertebrate, some arthropods (Dore et al., 2004) and dictyostelium (Hudson et al., 2005).

In vitro studies had revealed that DNA-PK can phosphorylate a number of nuclear, DNA binding proteins with supposed functions in transcription, replication, recombination and repair (Lees-Miller et al., 1992). The sites phosphorylated by DNA-PK were identified as serine and threonine that are immediately followed by a glutamine on the linear sequence; SQ/TQ (Lees-Miller et al., 1992), although there are a considerable number of exceptions reported. The protein phosphorylation by DNA-PK should be essential for NHEJ, as catalytically inactive form of DNA-PKcs can restore at most partial NHEJ activity to DNA-PKcs deficient cells (Kurimasa et al., 1999). However, it is presently unclear what is/are the *in vivo* phosphorylation target(s) essential for DNA repair.

Recent studies have shed light on the phosphorylation of DNA-PKcs itself. At least 16 sites of autophosphorylation have been identified (Chan et al., 2002; Douglas et al., 2002; Ding et al., 2003). Most of them are clustered within 2023 - 2056 (PQR cluster), 2609 – 2647 (ABCDE cluster) and 2671 – 2677. Some of them may be phosphorylated by ATM or ATR *in cellulo* (Chen et al., 2007; Yajima et al., 2006) It has been demonstrated that, *in vitro*, autophosphorylation of DNA-PK leads to loss of kinase activity and dissociation from Ku (Chan et al., 1996). It should be also noted that substitution of serines and threonines within ABCDE cluster with alanine results in greater radiation sensitivity than DNA-PKcs null cells and also in reduced rates of HR. Thus, autophosphorylation, especially within ABCDE cluster might regulate DNA-PK activity negatively or switch repair pathway from NHEJ to HR.

2.3.3 XRCC4-DNA ligase IV

XRCC4 was isolated and cloned from a human cDNA sequence whose expression in the XR-1 cells, derived from Chinese Hamster ovary and phenotypically similar to *scid* and *xrs,* conferred normal V(D)J recombination ability and also DSB repair activity (Li et al., 1995). Biochemical studies lead to finding that it is associated with DNA ligase IV (Critchlow et al., 1997; Grawunder et al., 1997). Mutations in DNA ligase IV gene have been identified in radiosensitive leukemia patient (Badie et al, 1995; Riballo et al., 1999) and in patients exhibiting developmental delay and immunodeficiency, which is called ligase IV syndrome (O'Driscoll et al., 2001). Although mutation in XRCC4 gene has not been found in humans,

there are some polymorphisms associated with colorectal cancer and childfood leukemia (Bau et al., 2010; Wu et al., 2010). Disruption of either XRCC4 or DNA Ligase IV gene in mice leads to embryonic lethality with a primary defect in neurogenesis and severe neuronal apoptosis (Barnes et al., 1998; Frank et al., 1998; Gao et al., 1998). Mutants of *DNL4* and *LIF1* genes, the yeast orthologue of human DNA Ligase IV and XRCC4, respectively, exhibited a phenotype similar to that of HDF1 and 2 mutants, indicating its role in recombination and repair (Wilson et al., 1997; Teo and Jackson, 1997, 2000).

XRCC4-DNA Ligase IV is a critical complex formed *in vivo* (Critchlow et al., 1997; Grawunder et al., 1997) for the ligation of the broken DNA ends via NHEJ pathway. The presence of XRCC4 stabilize and activates DNA Ligase IV (Grawunder et al., 1997; Bryans et al., 1999) by stimulating its adenylation which is the first chemical step in ligation (Modesti et al., 1999). XRCC4 forms a homodimer and associates with a polypeptide at the C-terminus of DNA Ligase IV (Critchlow et al., 1997; Junop et al., 2000; Sibanda et al., 2001). This interaction is mapped to the central coiled coil domain of XRCC4 and the inter BRCT linker region at the C-terminus of DNA Ligase IV. This region within DNA Ligase IV, termed as the XRCC4-interacting region (XIR) was deemed necessary and sufficient for XRCC4-Ligase IV interaction (Grawunder et al., 1998). Recently a high resolution crystal structure of human XRCC4 bound to the C-terminal tandem BRCT repeat of DNA Ligase IV was reported. It revealed an extensive binding interface formed by helix-loop-helix structure within the inter-BRCT linker region of Ligase IV, as well as significant interactions involving the second BRCT domain that induces a kink in the tail region of XRCC4 (Wu et al., 2009). This interaction was demonstrated as essential to stabilize the interaction between the XIR of DNA Ligase IV and XRCC4, while the first BRCT domain was considerably dispensable.

2.3.4 XLF/ cernunnos

Although above five factors had been identified by 1998, there were indications of the existence of additional factor essential for mammalian NHEJ. First, 2BN cell line, which is derived from radiosensitive and immunodeficient patient, showed defective NHEJ but all the known NHEJ components were normal. Second, in 2001, NEJ1/LIF2 was identified as a new essential factor of NHEJ in budding yeast (Kegel et al., 2001; Valencia et al., 2001; Ooi et al., 2001; Frank-Vaillant & Marcand, 2001).

XLF was identified in the yeast two hybrid screen for XRCC4 inteacting protein (Ahnesorg et al., 2006) and named XRCC4-like factor, as it was predicted to have 3D structure similar to that of XRCC4. It is also identified as Cernunnos missing in patients with growth retardation, microcephaly, immunodeficiency, increased cellular sensitivity to ionizing radiation and a defective V(D)J recombination (Buck et al, 2006). It is a 33kDa protein with 299 amino acid residues. NHEJ deficient 2BN cells lacked XLF due to a frameshift mutation (Ahnesorg, 2006). XLF was found to be a genuine homologue of Nej1p from budding yeast (Callebaut et al., 2006). XLF was also shown to be conserved across evolution (Hentges et al., 2006) and to be a paralogue of XRCC4 (Callebaut et al., 2006).

Chromatographic analyses established XLF existing as dimer and crystallographic studies demonstrated its interaction through globular head-to-head domain with that of XRCC4 (Andres et al., 2007; Li et al., 2008). Three-dimensional X-ray scattering characterized a tetramer formation of XRCC4, while the XRCC4-XLF interaction was still mediated through glogular head domains which rendered it suitable for DNA alignment and Ligase IV function (Hammel et al., 2010). XLF possesses DNA binding activity dependent on the

length of DNA (Lu et al., 2007a) and ability to ligate mismatched and non-cohesive ends (Tsai et al., 2007).

2.3.5 Processing enzymes

Pathologic and physiologic breaks create incompatible DNA ends which are not as easy to rejoin as those created *in vitro* by restriction enzyme digestion. It requires removal of excess DNA and fill-in of gaps and overhangs in order to make them compatible for the DNA ligase activity.

Artemis was identified as the causative gene for human RS-SCID (Moshous et al., 2001). Artemis forms a complex with DNA-PKcs and expresses 5′ to 3′ exonuclease activity and endonuclease and endonuclease activity at the junction of single-stranded and double-stranded DNA (Ma et al., 2002). Although, the signal joint formation during V(D)J recombination does not require Artemis or DNA-PKcs for joining, all of the components of NHEJ including Artemis are required for coding ends. Artemis in association with DNA-PKcs is deemed necessary for the opening of hairpin structures (Lu et al., 2007b). Artemis is phosphorylated both by DNA-PKcs and ATM (Poinsignon et al., 2004; Zhang et al., 2004).

Polymerases μ and λ belong to pol X family and might fill gaps and 5′overhangs (Ramadan et al., 2003). Polynucleotide kinase/phosphatase (PNKP) adds phosphate group to 5'-hydroxyl end and also removes phosphate group from 3′-phosphorylated end (Koch et al., 2004; Clements et al., 2004;Whitehouse et al., 2001). Aprataxin (APTX) is initially identified as the product of the gene defective in genetic disorder early-onset ataxia with oculomoter apraxia (Date et al., 2001) and later shown to remove AMP from abortive intermediates of ligation (Ahel et al., 2009). PNK- and APTX-like FHA protein (PALF, also known as APLF, C2orf13 or Xip1) has AP endonuclease activity (Kanno et al., 2007; Iles et al., 2007). Recent study showed that APLF also has histone chaperone activity (Mehrotra et al., 2011) and that it co-operates with PARP-3, which is newly found as a DSB sensor (Rulten et al., 2011). It might be noted that all of these factors bears BRCT or FHA domain as module to bind phosphorylated proteins. Polymerases μ and λ possess BRCT domain. PNKP, APTX and PALF possess FHA domain, which is structurally similar to each other and known to interact with CKII-phosphorylated XRCC1 or XRCC4 (see below).

2.4 Alternative NHEJ pathways

Apart from the classical NHEJ model, there are also studies by several groups highlighting NHEJ as a more sophisticated and complex mechanism involving a cross–talk between pathways including proteins other than DNA-PKcs, Ku, XRCC4-DNA Ligase IV.

2.4.1 ATM dependent pathway

Human genetic disorder, Ataxia Telangiectasia (AT) is caused by mutation in the ATM (Ataxia Telangiectasia mutated) gene and is characterized by chromosomal instability, immunodeficiency, radiosensitivity, defective cell cycle checkpoint activation and predisposition to cancer indicating its responsibility in genome surveillance (Jorgensen and Shiloh, 1996). ATM deficiency causes early embryonic lethality in Ku or DNA-PKcs deficient mice, thus providing NHEJ an independent role for the DNA-PK holoenzyme (Sekiguchi et al., 2001). ATM and Artemis, together with NBS1, Mre11 and 53BP1 function in a sub-pathway that repairs approximately 10% of DSBs, probably requiring end-processing (Riballo et al., 2004). Another study suggested three parallel, but mutually crosstalking,

pathways of NHEJ, *i.e.*, core pathway mediated by DNA-PKcs and Ku, ATM-Artemis pathway and 53BP1 pathway, all of which finally converge on XRCC4-DNA Ligase IV (Iwabuchi et al., 2006).

2.4.2 Back-up NHEJ pathway

Repair in IR-induced DSBs in higher eukaryotes is mainly dominated by NHEJ which is faster as compared to other mechanisms. However, it is severely compromised in case of defects in DNA-PKcs, Ku and DNA Ligase IV (DiBiase et al., 2000; Wang et al., 2001). An array of biochemical and genetic studies have shown that despite the prevalence of DNA-PK dependent pathway, cells deficient in either of its components are still able to rejoin a majority of DSBs, operating with slower kinetics, using an alternative pathway (Nevaldine et al., 1997; Wang et al., 2003). Chicken DT40 cells defective in HR rejoin IR induced DSBs with kinetics similar to those of other cells with much lower levels of HR. Nevertheless, rejoining of DSBs with slow kinetics is associated with incorrect DNA end-joining which is incompatible with the mechanism of HR (Löbrich et al., 1995). These observations led to the model that DNA DSBs are rejoined by two pathways, one of which is DNA-PK dependent (D-NHEJ) and an alternative pathway termed as Back-up (B-NHEJ) pathway (Wang et al., 2003) possibly prone to erroneous re-joining and utilization of microhomologies (Roth DB, 1986). Further investigations ascertained the role of DNA-PK in the functional co-ordination of D-NHEJ and B-NHEJ, suggesting that the binding of inactive DNA-PK to DNA ends not only blocks the D-NHEJ but also interferes with the function of B-NHEJ (Perrault et al., 2004). The DNA-PK and Ku complex is believed to recruit other repair proteins like XRCC4-DNA Ligase IV complex and stimulate the ligation of DNA ends (Ramsden and Gellert, 1998) in D-NHEJ pathway.

DNA Ligase IV deficient mouse embryonic fibroblasts retained significant DNA end-joining activity which was reduced upto 80% by knocking down DNA Ligase III. Thus DNA Ligase III was identified as a vital component of B-NHEJ (Wang et al., 2005). PARP-1 was initially pointed to bind to DSBs with a higher efficacy than to SSBs (Weinfeld et al., 1997) and with a greater affinity than that of DNA-PKcs (D'Silva et al., 1999). It has also been shown to interact with both the subunits of DNA-PK (Galande and Kohwi-Shigematsu, 1999; Ariumi et al., 1999) catalyzing their poly(ADP-ribosyl)ation (Li et al., 2004; Ruscetti et al., 1998). Using chemically potent producer of DSBs, calicheamicin γ1, a new mechanism was identified operating independently but complementing the classical NHEJ pathway. Proteins such as, PARP-1, XRCC1 and DNA Ligase III, which were believed to be otherwise involved in Base Excision Repair (Caldecott, 2003) and SSB repair (Caldecott, 2001) surmised a new mechanism encompassing synapsis and end-joining activity.

Above mentioned studies evidently illustrate alternative DNA end-joining pathways to contribute in the repair of DSBs in order to maintain the genomic integrity when D-NHEJ is compromised. However, due to their low fidelity, they are directly implicated in genomic instability (Ferguson et al., 2000), aberrant coding and signal joint formation during V(D)J recombination (Taccioli et al., 1993;Bogue et al., 1997) as well as formation of soft tissue sarcomas (Sharpless et al., 2001) that potentially leads to cancer.

3. Recruitment and assembly of NHEJ factors at DSB

The key players of NHEJ are named, but the mechanism of their recruitment and hierarchy of assembly on the DNA DSB is not yet well clarified. Many proteins in the HR pathway, *e.g.*, Nbs1-Mre11-Rad50, BRCA1 and Rad51, exhibit local accumulation after DSB induction,

forming microscopically visible structures, termed ionizing radiation-induced foci (IRIF) (Maser et al., 1997). Such change in the localization of HR proteins has been observed also in partial volume irradiation (Nelms et al., 1998) and laser micro-irradiation experiments (Kim et al., 2002). As the distribution of these proteins after irradiation, at least partially, overlapped with irradiated area or DSBs, visualized by DNA end labeling or immunofluorescence analysis of γ-H2AX, these phenomena are believed to reflect the accumulation of these proteins around DSB sites. In the case of NHEJ proteins, however, IRIF has been observed only for autophosphorylated form of DNA-PKcs (Chan et al., 2002). Recently, there are increasing number of studies using laser micro-irradiation demonstrating the accumulation of NHEJ molecules in irradiated area. Another approach to examine the association of DNA repair proteins with damaged DNA is sequential extraction with increasing concentration of detergent or salt.

3.1 Recruitment of XRCC4 to chromatin DNA in response to ionizing radiation

We employed sequential extraction with detergent-containing buffer to examine the binding of XRCC4 to DSB (Kamdar and Matsumoto, 2010). The retention of XRCC4 to subcellular fraction consisting of chromatin DNA and other nuclear matrix structures increased in response to irradiation. Micrococcal nuclease enzyme which specifically cleaves the chromatin DNA into smaller nucleosomal fragments revealed that XRCC4 is tethered to chromatin DNA after irradiation.

Through quantitative analyses, it was estimated that only one or few XRCC4 molecules might be recruited to each DNA end at the DSB site. This can be speculated based on the stoichiometric results depicting a complex consisting of two XRCC4 molecules forming a dimer and one Ligase IV molecule (Junop et al., 2000). The accumulation of XRCC4 on the damaged chromatin is very rapid and sensitive as the response after radiation is observed in $\leq$ 0.1hr and is stable until at least 4 hrs. This phenomenon is in parallel to the appearance of phosphorylation of H2AX which is observed as foci until the DSBs are repaired and then their disappearance from the resealed DNA (Svetlova et al., 2010). XRCC4 could be retained on the damaged chromatin as long as the repair complex carries out the rejoining of the DNA ends which pivotally includes ligation by XRCC4-DNA Ligase IV. In addition, the residence of XRCC4 on chromatin might be very transient, particularly after the irradiation with small and conventional doses. These observations can reasonably explain why it has been difficult to capture the movement of NHEJ enzymes to DSB sites.

Using a similar approach, the movement of NHEJ molecules in response to DSB induction by neocarzinostatin or bleomycin was reported (Drouet et al., 2005). Conversely, there are several differences between the results of the two studies. First, they observed that DNase I treatment released DNA-PKcs and Ku but not XRCC4 and DNA Ligase IV, leading to the idea that XRCC4 and DNA Ligase IV were bound to nuclear matrix or other structures rather than chromatin itself. In the present study, XRCC4 retained after buffer extraction could be released by micrococcal nuclease treatment, indicating its binding to chromatin DNA. Second, they mentioned that the movement of NHEJ molecules could be observed only after high doses of irradiation in their study. The present study has demonstrated small but significant increase in the chromatin binding of XRCC4 even after physiologically relevant dose, *i.e.*, 2Gy, of irradiation.

3.2 Phosphorylation of XRCC4

Several studies have shown that DNA-PK can phosphorylate XRCC4 *in vitro*, decreasing its interaction with DNA, although the significance of this phenomenon is presently

unclear (Critchlow et al., 1997; Leber et al., 1998; Modesti et al., 1999). Moreover, our research group demonstrated XRCC4 phosphorylation in living cells, which was induced by ionizing radiation in a manner dependent on DNA-PKcs (Matsumoto et al., 2000), indicating that XRCC4 is an i*n vivo* and not merely an *in vitro*, substrate of DNA-PK. However, the presence of DNA-PK did not seem as a pre-requisite for XRCC4 recruitment to chromatin as demonstrated by siRNA and specific kinase inhibitors against DNA-PKcs.

DNA-PK is autophosphorylated and leads to the phosphorylation events on the target proteins. An earlier study also detected XRCC4 on DNA ends in a phosphorylated form dependent on DNA-PK. However, phosphorylation was deemed dispensable for XRCC4-DNA Ligase IV loading at DNA ends since stable complexes involving DNA-PK and the ligation complex were recovered in the presence of wortmannin which is a PIKK inhibitor (Calsou et.al 2003). A recent study using laser irradiation demonstrated XRCC4 accumulation in irradiated area, which also did not require DNA-PKcs (Mari et al., 2006; Yano et al., 2008). All these observations in aggregate thus lead to the unanswered question as to what mechanism is involved in XRCC4 recruitment to damaged chromatin DNA.

Then, what is the importance of the phosphorylation of XRCC4, if any? It has been awaited to find the biological consequence of XRCC4 phosphorylation by DNA-PK through the identification and elimination of the phosphorylation site(s). Several groups, employing mass spectrometry, identified Ser260 and Ser318 as the major phosphorylation sites in XRCC4 by DNA-PK *in vitro* (Lee et al., 2002; Yu et al., 2003; Lee et al., 2003; Wang et al., 2004). However, it is presently unclear whether these sites are phosphorylated in living cells, especially, in response to DNA damage. Furthermore, the mutants lacking these phosphorylation sites appeared fully competent in the restoration of radioresistance and V(D)J recombination in CHO-derived XRCC4-deficient XR-1 cells and also exhibited normal activity in DNA joining reaction in cell-free system, leading to the conclusion that XRCC4 phosphorylation by DNA-PK was unnecessary for these functions (Lee et.al 2003; Yu et.al 2003). However, our group recently identified four additional phosphorylation sites in XRCC4 by DNA-PK and found that at least three of them would be important for DSB repair, because disruption of these sites resulted in elevated radiosensitivity (Sharma, Matsumoto et al., unpublished results).

3.3 Recruitment dynamics of NHEJ complex on damaged chromatin

XRCC4 associates in a tight complex with DNA Ligase IV. XRCC4 is essential for the stability of ligase IV in mammalian cells (Bryans et.al 1999). It also initiates the chemical reaction of ligation reaction by bringing about the adenylation on Ligase IV to rejoin the DNA. Radiation induced modification, i.e phosphorylation of XRCC4 is also observed in the cells harboring the ligase IV gene. Although, it is evident from the above reports that phosphorylation is not a necessary phenomenon required for XRCC4 recruitment to chromatin, it occurs as a modification induced in response to radiation. These observations lead to two possible hierarchies; (a) ionizing radiation induces phosphorylation on DNA-PKcs which then in turn phosphorylates XRCC4 and the phosphorylated form is recruited to DSBs or (b) ionizing radiation stimulates XRCC4 recruitment to DSBs, chaperoned by other factors like ligase IV, and also recruitment of DNA-PKcs independently and then the kinase would bring about the phosphorylation

events. However, since current evidences render phosphorylation dispensable for recruitment of XRCC4, the second mechanism may seem more plausible.

Moreover, movement of DNA-PKcs to chromatin DNA is also diminished in the absence of DNA Ligase IV and Ku. In addition, structural and crystallographic studies have displayed that the interaction between XRCC4 dimer and DNA Ligase IV is via the linker region on ligase IV between the tandem BRCT domains (Grawunder et al., 1998). A recent high resolution crystallographic study has revealed an extensive DNA Ligase IV binding interface for XRCC4 forming a helix-loop-helix structure forming a clamp within the inter-BRCT linker region. This loop buries and packs against a large hydrophobic surface of XRCC4, thus inducing a kink in the tail region of XRCC4, thereby involving numerous interactions between the BRCT2 domain of ligase IV and XRCC4 which are expected to play a major role in the interactions between the two proteins (Wu et.al., 2009). Mutational analysis in several of these hydrophobic residues would give a better insight in the mode of interaction alterating the conformation of both the molecules for recruitment on DNA ends.

XLF or Cernnunos is also considered a vital component of the ligation complex to reseal the DNA ends. XLF has been demonstrated to interact with XRCC4 via the globular head domains at the amino-terminal region of both the proteins forming a heterodimeric structure (Andres et al., 2007). The response to ionizing radiation could thus be expected to be similar to that evoked in XRCC4. Conversely, the protein was not found to be tethered to chromatin even after extraction with a high detergent concentration. Contrasting to that observed in case of XRCC4, XLF accumulation was neither rapid or transient nor sensitive to be observed at conventional radiation dose. This leads to the possibility that XLF association to XRCC4 is highly unstable and does not directly adhere to chromatin structures. A parallel observation was drawn by another study wherein they demonstrated that XRCC4 was dispensable for XLF recruitment to DSBs, although it could act as a stabilizing factor and cause a dynamic exchange between the free and bound protein once XLF is recruited on the DNA free ends (Yano et.al., 2008). Very recent study indicated that 10 amino acid region at the C-terminal of XLF is essential for interaction with Ku and for recruitment to DSB (Yano et al., 2011).

Intriguingly, transgenetically expressed XLF protein demonstrated a similar trend, except that the retention was observed in the subcellular nucleosolic fraction, alleged as tethered to chromatin. This disparity in the observation can be attributed to the difference in behaviour between endogenous and exogenously expressed molecules.

Owing to the recruitment of XRCC4 during the inhibition of phosphorylation by the kinases, a possible speculation leads to the idea that either or both of ligase IV and XLF molecules could play a role as a chaperone responsible for the recruitment of XRCC4 to damaged chromatin.

Live cell imaging studies have demonstrated that Ku recruits XLF and is also likely to mediate the XLF-DNA interaction (Yano et al., 2008). Therefore, the vital component of NHEJ, Ku might be mediating the interaction between XRCC4 and DSB via DNA Ligase IV or also between Ligase IV and DSB via XRCC4, though ligase IV possess a DNA-binding region at the N-terminus.

Another very intriguing analysis has exhibited that PARP-3, whose function was previously unknown, accumulates APLF (Aprataxin-like factor) to the site of DSBs which in turn supports the retention of XRCC4-DNA Ligase IV on the chromatin (Rulten et al., 2011).

Another possibility is that XRCC4 moves to a DSB site autonomously due to its intrinsic DNA end-binding activity (Modesti et al., 1999). Furthermore, XRCC4 was shown to interact with polynucleotide kinase (PNK) (Koch et al., 2004) or aprataxin (APTX) (Clements et al., 2004), depending on the phosphorylation by casein kinase II. Unexpectedly, unphosphorylated XRCC4 interacts with PNKP, although with a lower affinity, but CKII mediated XRCC4 phosphorylation inhibited the PNKP activity (Mani et al., 2010). In addition, XRCC4 has been shown to undergo monoubiquitination (Foster et al., 2006) and SUMOylation (Yurchenko et al., 2006), the former of which was shown to be DNA damage-inducible. The role of such posttranslational modifications on the chromatin-recruitment of XRCC4 is of another interest.

Additionally, studies by several groups have suggested that NHEJ is more sophisticated than thought initially and involves many proteins other than DNA-PKcs, Ku, XRCC4-DNA ligase IV, XLF/Cernunnos. In order to investigate into the entirety of the complex compounding several molecules from NHEJ and particularly from other repair or physiological pathways: XRCC4 associated complex bound to chromatin, supposedly at the last step of resealing the DNA nicks and gaps, can be isolated and analysed.

One of the other speculations is that the unwinding may be carried out by Ku since it possesses helicase activity in an ATP dependent manner (Blier et al., 1993) and is supposedly the earliest protein in repair hierarchy. Certain studies have shown a functional interaction between the Ku heterodimer and WRN (Karmakar et al., 2002) emphasizing its significance in DNA repair and metabolism pathways. The exonuclease but not the helicase activity of WRN is stimulated by physical interaction with XRCC4-ligase IV (Kusumoto et al., 2008).

ATM and Artemis, together with Nbs1, Mre11 and 53BP1, function in a subpathway of NHEJ that repairs approximately 10% of DSBs, probably those require DNA end processing (Riballo et. al, 2004). Another study suggested three parallel, but mutually crosstalking, pathways of NHEJ, *i.e.*, core pathway mediated by DNA-PKcs and Ku, ATM-Artemis pathway and 53BP1 pathway, all of which finally converge on XRCC4-DNA ligase IV (Iwabuchi et al., 2006). Recent studies indicated the requirement of chromatin remodeling factors, like ALC1 and ACF1, for the recruitment of NHEJ molecules to DSB (Ahel et al., 2009; Lan et al., 2010).

It will be of interest to investigate whether all of the above mentioned proteins play some role in the recruitment of XRCC4-DNA Ligase IV to DSB sites or, conversely, are recruited to DSB sites through interaction with XRCC4. This entire conglomerate of proteins has yet to reveal complex mechanisms and cross-talk between other repair and cellular pathways.

These questions may be addressed by examining the chromatin-recruitment of deletion or point mutants of XRCC4 and by applying siRNA or inhibitors of the above listed molecules in experimental systems. They could then be optimized for use as adjuvants in radiotherapy.

Proteomic analysis is one of the vital instruments to examine any kinase network involving *in vivo* substrates. Such modern technologies have helped to understand that the DNA damage repair response is much sophisticated and complicated than anticipated earlier. It connects NHEJ with chromatin remodelling as well as transcription processes which are also pivotal to cellular functions; thereby aspiring to investigate the cross-talks involved in the repair mechanics.

3.4 Future perspectives

There have been several studies including ours, demonstrating various mechanisms for the dynamics and assembly of the repair machinery on the damaged DNA site in response to various forms of endogenous and exogenous stress. A certain study also suggests that the DNA damage response does not require the DNA damage but the stable association of the repair factors for a prolonged period of time with chromatin which is likely a critical step in triggering, amplifying and maintaining the DNA damage response signal (Soutoglou and Misteli, 2008). It will thus be interesting to investigate the capricious questions as to what are the exact signalling mechanisms to trigger the DSB repair response or the role of several macromolecules involved in different cellular processes. Thus, the assembly of non-homologous end joining protein complex at DSB was not as simple as thought in classical models and further studies are warranted to fully elucidate the processes. Another important aspect, not clarified, is to understand the hierarchy and mechanism of the disassembly of the repair machinery, involved in NHEJ or from cross-talk pathways, from the site of refurbished DNA. Finally, understanding the mechanisms of DNA repair at molecular levels might bring us a new approach to be applied in cancer radiotherapy or chemotherapy.

4. Conclusions

DSB repair through NHEJ has been considered rather simple reaction, basically comprised of six core factors, Ku70, Ku80, DNA-PKcs, XRCC4, DNA Ligase IV and XLF. However, the mechanism how these molecules are recruited to DSBs and assembled into repair machinery is not fully understood. It has been difficult even to observe the recruitement of NHEJ molecules by immunofluorescence or simple labeling with fluorescent proteins. However, laser microirradiation technique combined with fluorescent protein and biochemical fractionation enabled us to capture the binding of NHEJ factors to DSBs. NHEJ would involve a number of processing enzymes, whose function or regulation is largely unclear. Additionally, most recent study shed light on the importance of chromatin remodeling prior to the binding of Ku. Obviously, further studies are warranted to elucidate this complexity.

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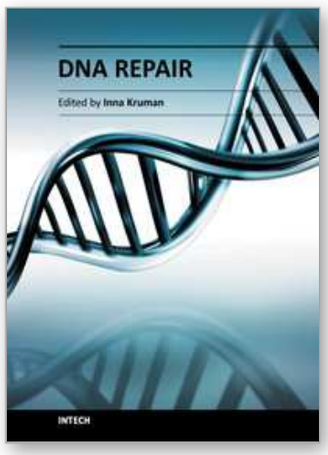

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The book consists of 31 chapters, divided into six parts. Each chapter is written by one or several experts in the corresponding area. The scope of the book varies from the DNA damage response and DNA repair mechanisms to evolutionary aspects of DNA repair, providing a snapshot of current understanding of the DNA repair processes. A collection of articles presented by active and laboratory-based investigators provides a clear understanding of the recent advances in the field of DNA repair.

How to reference

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Radhika Pankaj Kamdar and Yoshihisa Matsumoto (2011). DNA Double-Strand Break Repair Through Non-Homologous End-Joining: Recruitment and Assembly of the Players, DNA Repair, Dr. Inna Kruman (Ed.), ISBN: 978-953-307-697-3, InTech, Available from: http://www.intechopen.com/books/dna-repair/dna-double-strand-break-repair-through-non-homologous-end-joining-recruitment-and-assembly-of-the-pl

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