Chapter 19

Repair of Double-Strand Breaks by Nonhomologous End Joining: Its Components and Their Function

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Chapter Outline

1. INTRODUCTION

The genomic DNA is exposed to a continuous endogenous and exogenous damage through reactive oxygen species (ROS), chemicals, viral infections, replication errors, and ionizing radiation. The large variety of DNA-damaging agents causes a large variety of DNA damage, and thus, necessitates distinct and specialized DNA-repair pathways to guard genomic stability. This chapter will exclusively focus on double-strand breaks (DSBs) and their repair by nonhomologous end joining (NHEJ).

DSBs represent the most dangerous DNA damage and a single unrepaired break is sufficient to induce cell death. Nonetheless, hematopoietic cells undergo programmed DSBs during V(D)J and class switch recombination (CSR) to insure infinite variability of antibodies and T-cell receptors (TCRs) matching each invading pathogenic microorganism. Regardless whether DSBs are of toxic or programmed origin, inability to repair these breaks may cause either cell death or chromosomal rearrangements, which may lead to malignant transformation, often with deleterious consequences. Therefore, to minimize the chance of this catastrophic event, an immediate and faithful repair of DNA DSBs is demanded.

Dividing cells unpack and replicate genomic DNA making it particularly susceptible to previously mentioned damage. This poses a high risk of chromosomal aberration that could be passed to descending cells. Thus, to meet the cell cycle-specific requirements and preserve genomic stability, evolutionary two distinct DNA DSB-repair pathways have evolved. Homologous recombination (HR), a high-fidelity mechanism, which functions predominantly in the S and G2 phase of the cell cycle and utilizes the sister chromatid as a template to restore identical copy of the damaged DNA. In contrast, NHEJ is mainly prevalent in the G0 and G1 phase of the cell cycle and joins broken DNA ends without template. Recently, there are growing evidences that NHEJ consists of two pathways, the classical NHEJ (C-NHEJ) and the alternative NHEJ (A-NHEJ). The latter exhibits high mutagenic propensity, whereas the former is characterized by the ability to join DNA ends directly or with minimal processing.

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Here, the current knowledge of NHEJ will be summarized. It will be distinguished between the classical and alternative NHEJ pathway. Components of each pathway will be briefly introduced emphasizing their most important characteristics, such as structure, function, binding partners, and possible regulation. Common and distinct phenotypic changes in case of mutation or loss of NHEJ components will be pointed out. The role of C-NHEJ as an integral part of V(D)J and class switch recombination will be discussed.

2. CLASSICAL NHEJ

The C-NHEJ pathway of DSB repair is the major DSB-repair pathway in mammalian cells and has been already extensively studied for many years. The initial studies came from the laboratory of F. Alt, which described two nonlymphoid-specific genes involved in DNA repair, later identified as Ku80 und Xrcc4. Subsequently, over many years altogether eight components of C-NHEJ have been identified; four highly conserved Ku70, Ku80, Xrcc4, and Lig4 and three accessory units Artemis, XLF, and DNA-Pkcs (Fig. 19.1). In addition, PAXX protein, a paralog of XLF and Xrcc4, was reported in 2015 as potential component of C-NHEJ [1]. Defects in C-NHEJ lead to some characteristic phenotypic changes, such as radiosensitive severe combined immunodeficiency (RS-SCID), premature aging, microcephaly and growth retardation [2,3].

In undamaged cells, components of C-NHEJ are disassembled and a single DSB is sufficient to activate the assembly of a functional C-NHEJ complex at the site of damage to seal the break. The repair of a DSB is extremely complex and progresses through four successive phases: sensing and tethering, end processing, ligation of broken DNA ends, and finally dissociation of the C-NHEJ complex from resolved break (Fig. 19.1). In contrast, components mediating these steps functionally overlap these phases. For instance, the Ku70/80 heterodimer is not only able to sense and tether breaks, but it also removes abasic nucleotides from broken ends.

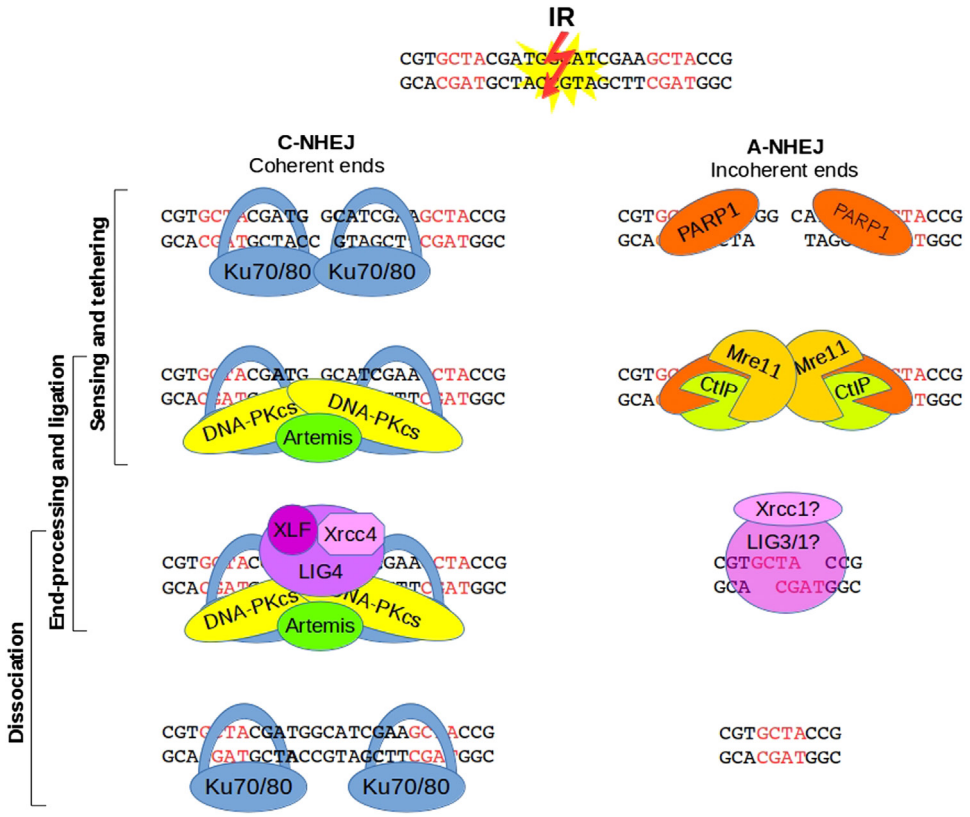

FIGURE 19.1 Cartoon representing NHEJ-mediated repair. Repair of coherent ends (on the left) is mediated by the classical NHEJ, whereas alternative NHEJ (on the right) mediates repair of non-coherent breaks. C-NHEJ: Ku70/80 heterodimers form a basket-like structure and instantly enclose broken DNA ends. Concurrently, Ku70/80 complex induces sequential assembly of functional C-NHEJ complex that ligates the breaks. Upon resolution of the break Ku70/80 complex remains trapped on linear DNA. The escape mechanism is not clear, degradation of Ku70 and Ku80 proteins is considered. A-NHEJ: PARP1 senses the DSB and possibly recruits Mre11 and CtIP nucleases to carry out limited resection (see also Fig. 19.4). This resection proceeds until microhomologies (highlighted with red) are found. The complementary overhangs hybridize and LIG3/1 seals the break. The phases are presented as overlapping to emphasize the multifaceted role of each component.

The first phase is initiated by Ku70/80 complex, which binds with high affinity to broken DNA ends and tethers them together. To execute this role, Ku70 and Ku80 form a ring structure suitable to accommodate DNA ends. Ku70/80 heterodimers associate with the tip of broken ends and anchor each other to hold opposite DNA ends in close vicinity. Concomitantly, Ku70/Ku80 complex serves as a docking platform for DNA-PKcs and other proteins involved in many aspects of DNA-damage response. Ku70/80 together with DNA-PKcs form a functional complex designated as DNA-PK [4].

Interaction of DNA-PKcs with Ku70/80 complex causes the latter to slide inwards on the DNA ends. The DNA-PKcs moves to the center of the synapsis and takes over the tethering of the DNA ends as well as stabilizes the entire DNA-PK/DNA synapsis. Reciprocally, this interaction induces conformational change of DNA-PKcs that activates the kinase domain. The catalytic activation of DNA-PKcs results in its autophosphorylation as well as phosphorylation of large array of proteins, among other C-NHEJ components [5,6].

Frequently, ends of DSBs are ragged and incompatible to undergo direct ligation. Thus, in the second phase, DNA ends are exposed to processing through specialized enzymes. To remove damaged or excessive nucleotides, nucleases trim DNA ends, whereas polymerases can fill in gaps, which arise when only partially complementary ends are annealed. Artemis is one of such nucleases and is recruited to DSB concurrently with DNA-PKcs. This enzyme cuts single-stranded DNA overhangs and opens hairpin structures. Polymerases lambda and mu (Pol λ and Pol μ, respectively) have been implicated in the gap filling during C-NHEJ-mediated repair, possibly they contribute to end retention [7,8].

The third phase is carried out by LIG4 complex consisting of DNA-ligase 4 (Lig4), Xrcc4, and XLF. This complex is recruited to the break by interaction with DNA-PK and restores the integrity of DNA molecule by covalently sealing two DNA ends. Once the break is resolved, in the fourth phase, C-NHEJ complex must dissociate from the DNA, a process that has been poorly studied. Possibly some of the components simply dissociate upon secondary modification, such as phosphorylation and may be recycled, whereas some others undergo ubiquitin-mediated degradation [7].

2.1 Components of Classical NHEJ

2.1.1 DNA-PK (DNA-Protein Kinase Catalytic Subunit/Ku70/Ku80) Complex

2.1.1.1 Ku70/80 Heterodimer

Ku70 and Ku80 make up the Ku70/80 heterodimer. They are encoded by the Xrcc5 and Xrcc6 genes, respectively, and are highly abundant in both prokaryotes and eukaryotes. The stability of these proteins depends on each other. Mice deficient in Ku70 show severely reduced expression of Ku80 and vice versa [9,10]. In eukaryotic cells, both Ku proteins consist of three domains (Fig. 19.2): an N-terminal von Willebrand A domain (vWA), a central DNA-binding domain, and a diverged C-terminal domain (CTD). Role of the vWA domain is poorly characterized but based on its homology to other proteins, it is thought to function as a protein–protein interaction site. The Ku80 CTD is only present in higher eukaryotes and is well established to interact with the DNA-PKcs, proximal to its kinase domain [5,11]. In lower eukaryotes lacking DNA-PKcs this domain is not present. The Ku70 CTD contains a distal SAP domain (SAF-A/B, Acinus, and PIAS motifs), which seems to increase binding to dsDNA and may also interact with other proteins [12,13].

Crystallographic structure of human Ku70/80 heterodimers has revealed that both proteins interact with each other through the central DNA-binding domain. These domains intertwine with each other to form an asymmetric ring structure, which avidly binds linear dsDNA as well as with lower-affinity hairpin ends in a sequence-independent manner. This is attributed to the inner structure of the ring that is lined with positively charged amino acid residues. These residues exclusively interact with dsDNA sugar–phosphate backbone and are able to accommodate about 14 base pair [14].

Simplified, Ku70/80 complex is a sensor of DSBs that initiates the assembly of C-NHEJ complex to restore integrity of a DNA molecule. In fact, the role of Ku70/80 proteins is highly complex and integrates different aspects of DNA repair. Besides sensing, Ku70/80 complex possibly tethers broken DNA ends keeping them in close vicinity for end-processing and subsequent ligation. Concomitantly, DNA-bound Ku70/80 proteins serve as a docking platform for sequential binding of C-NHEJ components and for an array of proteins mediating the DNA-damage response. Further, Ku70/80 have been reported to coordinate DSB repair with cell cycle arrest, pathway choice, and, if needed, apoptosis [4]. Interestingly, Roberts et al. published that Ku70 possesses 5′-dRP/AP lyase activity and is possibly involved in end-processing by removing 5′-apurinic and 5′-apyrimidinic (AP) sites [15].

Broken DNA ends are threaded through the eyelet-like structure formed by the Ku70/80 dimer and once the break is resolved, Ku70/80 remains trapped on the linear dsDNA molecule (Fig. 19.1). The escape mechanism of Ku70/80 complex from the DNA thread has been poorly studied and is not clear [4,7]. Based on the crystallographic structure, a conformational change leading to an opening of the eyelet is not possible [14]. However, there are some evidences for alternative

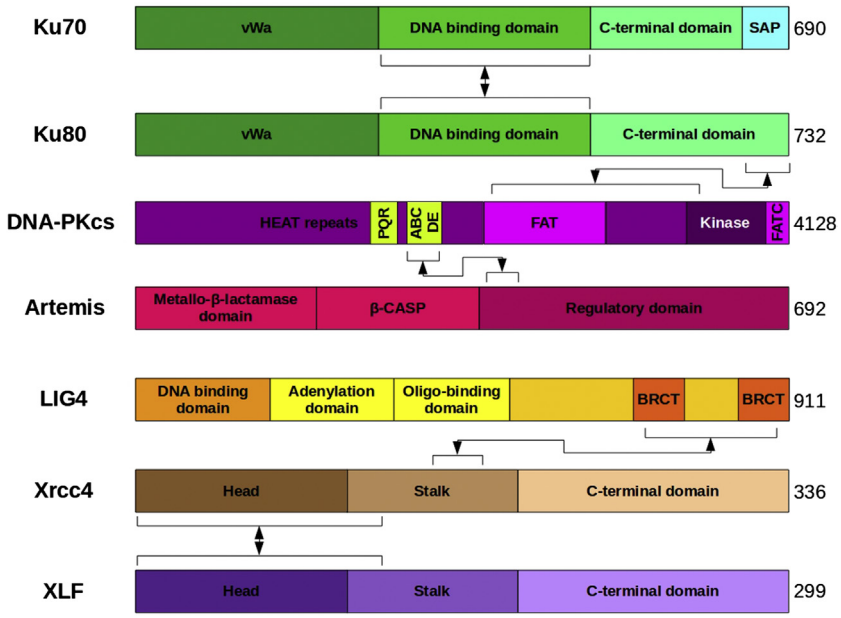

FIGURE 19.2 Cartoon representing C-NHEJ components. Indicated are main domains and possible interaction sites. *Summarized information is adopted from [4–6,24,36,38,45,55].*

mechanisms, such as DNA nicking or protein degradation. DNA nicking has been reported in yeast, but there are so far no studies supporting this mechanism in human. In contrast, RNF8 (RING finder protein 8) has been shown to ubiquitinate human Ku80 and to induce thereby its degradation. Depletion of RNF8 resulted in prolonged retention of Ku80 at the site of DNA damage and as a consequence NHEJ was impaired [4,16].

Mice deficient in Ku70 or Ku80 are viable, fertile, and show a strongly similar phenotype, such as growth retardation and SCID due to inability to rejoin V, D, and J segments. Surprisingly, in Ku70-deficient animals there is a residual development of T cells but it is not clear how these cells circumvent V(D)J recombination, a step that is absolutely indispensable for B- and T-cell development. Ku80 deficiency has no predisposition for tumorigenesis but in contrast, Ku70-deficient animals are significantly prone to develop thymic lymphomas. MEF cells isolated from Ku70- or Ku80-deficient animals exhibit radiosensitivity, intact DNA-damage checkpoints, and commit to premature senescence likely due to accumulation of DNA damage [9,10,17]. In humans, neither deficiency of Ku70 nor of Ku80 has been reported so far, most likely because deficiency is lethal. This speculation is further supported by the work of Wang et al., who demonstrated in human cells that Ku80 represses lethal telomere deletion [18]. The role of Ku70/80 in telomere maintenance is further supported in mice studies; however, these studies have provided conflicting results and the functional role in this process remains still to be determined [19,20].

2.1.1.2 DNA-PKcs (DNA-Dependent Protein Kinase Catalytic Subunit)

DNA-PKcs belongs together with ATM (ataxia telangiectasia related) and ATR (ataxia telangiectasia and Rad3 related) to the family of PIKK (phophatidyl inositol-3 kinase-like protein kinase) protein kinases, which mediate DNA-damage response and share overall a common structure. DNA-PKcs is a huge protein composed of 4128 amino acids corresponding to a molecular weight of about 465 kDa. The N-terminal domain is predicted to consist of HEAT repeats [Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), yeast kinase TOR1] and encompasses two-third of the entire protein (Fig. 19.2). Within this region are located major phosphorylation sites: PQR and ABCDE cluster. The C-terminus contains the catalytic kinase domain and two FAT domains (FRAP, ATM, TRAP) [5].

DNA-PKcs is the catalytic unit of the heterotrimer DNA-PK consisting of Ku70, Ku80, and DNA-Pkcs. Assembly of DNA-PK requires the binding of Ku70/80 complex to linear dsDNA ends, otherwise DNA-PKcs stays dissociated [21]. In this complex DNA-PKcs fulfills multiple tasks. The interaction between the DNA-PKcs and the Ku70/80 complex causes the latter to slide inward on each broken dsDNA end bringing the DNA-PKcs in a central position able to tether the DSB [22,23]. Reciprocally, the interaction with Ku70/80 complex induces conformational change of DNA-PKcs resulting in its catalytic activation and enables recruitment of subsequent C-NHEJ components [5,24].

Acquiring of catalytic activity by DNA-PKcs is of particular importance in C-NHEJ, though the exact role of target phosphorylation is poorly understood. Deficiency of kinase activity causes radiosensitivity and inability to rejoin V, D,

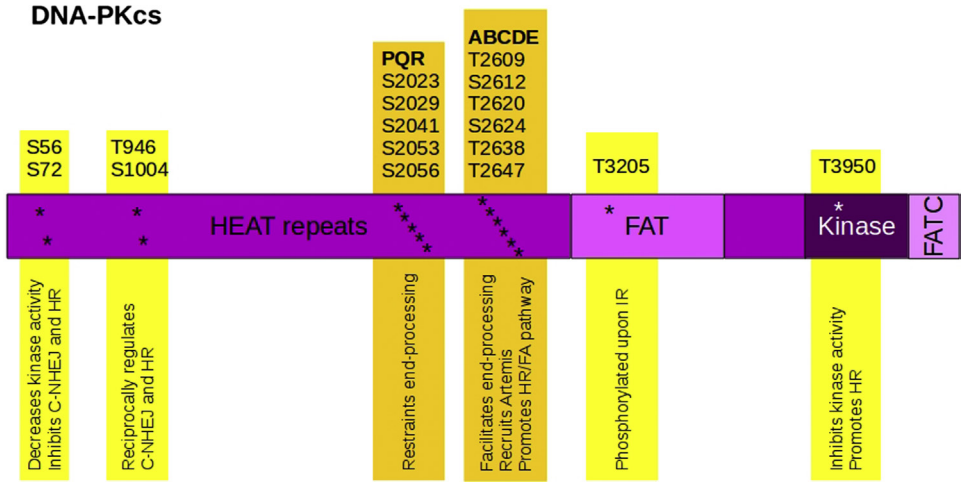

FIGURE 19.3 **Cartoon representing DNA-PKcs.** Highlighted are domains of DNA-PKcs and some phosphorylation sites. *Summarized information is adopted from reviews [4–6,24].*

and J segments [25]. However, phosphorylation of C-NHEJ components, such as Ku70, Ku80, Artemis, Xrcc4, and XLF seems to be redundant and does not affect C-NHEJ [24]. In contrast, phosphorylation of DNA-PKcs by the catalytic subunit itself and/or other kinases, such as ATM has been implicated in highly complex processes including regulation of end-processing, DNA-repair pathway choice, C-NHEJ complex dissociation, and auto-inactivation of kinase activity [5,7,24].

Altogether more than 40 sites of DNA-PKcs have been identified to undergo phosphorylation. Mutational analysis of both PQR and ABCDE cluster revealed that phosphorylation of these clusters do not affect the kinase activity but may reciprocally regulate end processing of dsDNA ends. The latter cluster seems to facilitate end processing and may promote a switch to the HR/FA (Fanconi) DNA-repair pathway, whereas PQC cluster is likely to counteract this process [24,26,27]. In contrast, phosphorylation of N-terminal cluster at serine 56 and 57 impacts kinase activity and blocks both C-NHEJ and HR. Phosphorylation of threonine 946 and serine 1004 does not affect kinase activity but it promotes HR. In line, phosphorylation of C-terminal threonine 3950 promotes HR and it also inhibits kinase activity. Threonine 3205 is phosphorylated in response to IR. Possibly differential phosphorylation of DNA-PKcs may be involved in fine-tuning of pathway choice (Fig. 19.3) [28,29].

DNA-PKcs knockout mice are viable but severely immunocompromised due to inability to carry out rejoining of V, D, and J segments and develop thymic lymphoblastic lymphomas. In contrast, mice expressing DNA-PKcs mutated in ABCDE cluster die prematurely from severe congenital bone marrow failure. This particularly severe phenotype is possibly attributed to the block of HR/FA (Fanconi) DNA-repair pathway resulting in apoptosis of hematopoietic stem cells [27,30,31].

At present, there are no reports on human deficient in DNA-PKcs but a missense mutation at position L3062R has been reported. In consequence, this patient exhibited a classical RS-SCID. On molecular basis, this was attributed to a functional failure in V(D)J recombination due to impaired activation of Artemis [32].

2.1.1.3 Artemis

Artemis was first identified in human patients suffering from RS-SCID. Alignment of its protein sequence revealed N-terminal catalytic domain encompassing metallo-β-lactamase and β-CASP subdomains and regulator CTD specific to Artemis (Fig. 19.2). Both N-terminal subdomains are conserved in nucleic acid processing enzyme belonging to the superfamily of metallo-β-lactamases [33,34]. Artemis is an endonuclease processing hairpin coding ends during V(D)J recombination and single-strand overhangs of dsDNA. As expected, Artemis deficiency in hematopoietic cells causes accumulation of unopened hairpins at the coding ends, whereas non-hematopoietic cells have increased sensitivity to ionizing radiation [35,36].

Recruitment of Artemis to a DSB as well as its subsequent catalytic activation is mediated through an interaction with DNA-PKcs. The interaction sites between both proteins are not well established. From the site of DNA-PKcs, the ABCDE cluster and its phosphorylation have be implicated in binding to the C-terminal region of Artemis. This site of Artemis is also phosphorylated by DNA-PKcs but its function remains to be determined [36,37].

2.1.2 LIG4 (DNA-Ligase 4/Xrcc4/XLF) Complex

LIG4 complex is composed of the DNA-Ligase 4, Xrcc4, and XLF and mediates the final step of the C-NHEJ repair. LIG4 is the catalytic unit of the complex capable of covalently sealing the ends of a DSB, whereas Xrcc4 and XLF do not show catalytic activity and rather play structural role. Both Xrcc4 and XLF form stable dimers by interaction between the head domains and proximal stalk regions (Fig. 19.2). These domains share structural but no sequence homology [38]. Xrcc4 is absolutely required for the stability of LIG4. Cells deficient in Xrcc4 do not exhibit LIG4 activity and the protein is not detectable suggesting rapid LIG4 degradation [39]. In contrast, XLF does not impact the stability of LIG4 or Xrcc4 [40,41]. The functional roles of both Xrcc4 and XLF proteins in LIG4 complex are not clear but both have been reported to stimulate LIG4 activity, possibly by promoting its adenylation [42,43]. Interestingly, XLF has been shown to particularly facilitate joining of non-cohesive ends [38,41,44].

In higher eukaryotes, there are three DNA ligases (LIG1, LIG3, and LIG4), which participate in different DNA-repair pathways [45]. They share a highly conserved DBD (DNA-binding domain) and a CD (catalytic domain) comprising of a nucleotidyl transferase and oligonucleotide/oligosaccharide-binding subdomain. Unique to LIG4 is the long C-terminal tail, which accommodates two BRCT (BRCA1 C-terminal) domains separated by a linker region. This region and the both BRCT domains are involved in binding of Xrcc4 [46,47]. Another unique feature to LIG4 is the catalytic ability to join non-cohesive ends containing gaps and the exclusive function only to participate in the C-NHEJ-mediated DNA repair [44]. In contrast, LIG1 and LIG3 are promiscuous and engage in distinct DNA-repair pathways and their function is shortly discussed in the context of A-NHEJ.

LIG4 catalyzes a multistep reaction requiring ATP to activate lysine K273 (in human) of the catalytic domain. The activated lysine carries AMP that is then transferred to 5′-PO4 of DNA to form activated 5′-AMP-DNA. Subsequently, the 3′-OH of a second DNA strand attacks the 5′-PO4 of the activated DNA releasing AMP and covalently sealing DNA strands. While this function is undisputed, the entire role of LIG4 complex in active NHEJ machinery is not clear [45].

Recruitment of LIG4 complex to the site of DNA damage is initiated by DNA-PK. But, there are emerging evidences that LIG4 complex itself may also contribute to assembly of a functional C-NHEJ complex serving as docking platform for end-processing enzymes. Studies carried out by Budman et al. clearly demonstrate that LIG4 complex is required for end processing of non-cohesive DNA ends [48]. The authors proposed a very attractive model, which implies that LIG4 complex binds to a break before end-processing enzymes, such as nucleases and polymerases and examines the nature of the break. Cohesive ends, which do not need end-processing, are directly ligated, whereas in case of non-cohesive ends, LIG4 complex recruits nucleases and polymerases. Once the ends are processed and made cohesive, LIG4 immediately seals the break protecting the DNA ends from further degradation [48,49].

As expected, targeted disruption of LIG4 or Xrcc4 in mice results in a strongly similar phenotype characterized by embryonic lethality due to massive neural apoptosis. Further, these animals suffer from arrested lymphocytosis through inability to rejoin V, D, and J segments and multiple other defects. MEF cells of these animals are markedly sensitive to IR [50,51]. Partially, the phenotype of LIG4 deficiency can be rescued by concomitant ablation of p53 or ATM. This is the case for embryonic lethality but not for arrested lymphocytosis. Rescue of lethality in LIG4/p53 or LIG4/ATM knockouts is not attributed to an improved or compensatory DNA-repair capacity, but rather due to attenuated response to DNA damage, and thus, diminished induction of neural apoptosis. In consequence, the cells bear to accumulate DNA damage but still fail to repair DSB or to rejoin V, D, and J segments [52,53]. In contrast, XLF null mice are viable, of normal size, and surprisingly, undergo almost unaffected V(D)J recombination, suggesting redundancy for this component in murine cells. Nonetheless, MEF cells show still increased sensitivity to IR [54].

In mouse, the LIG4 or Xrcc4 deficiency is not compatible with life and as expected, no deficiency in humans have been reported either. But, there are several reports of humans carrying mutations in LIG4, Xrcc4, and XLF genes. In case of LIG4, the severity of phenotype correlates with the residual LIG4 catalytic activity. These patients are generally characterized by sensitivity to radiation, features related to neural apoptosis, such as microcephaly and accordingly mental retardation, growth retardation, facial dysmorphisms, and skin abnormalities. Further, affected individuals show variable degrees of immunodeficiency (RS-SCID) [55]. Likewise, XLF or Xrcc4-affected patients show in general similar features, such as microcephaly, growth retardation, and radiosensitivity. But in striking contrast to LIG4 and XLF mutations, no immunodeficiency has been reported in patients carrying Xrcc4 mutations. This suggest that Xrcc4 may be redundant for V(D)J recombination in humans [40,41,56–61].

2.2 Programmed Double-Strand Breaks

To defend from continuously invading pathogens, higher vertebrates have developed an adaptive immune system composed of B and T cells. These cells have been equipped in a unique ability to tailor pathogen-specific immunoglobulins (Ig) and

TCRs. To do so, maturating B and T cells commit to highly dangerous programmed DSBs during V(D)J and CSR that, if not adequately repaired, result either in cell death or in chromosomal translocation [3,62].

V(D)J recombination occurs in both B and T cells and is initiated when the endonuclease RAG (recombination activating gene) binds to recombination signal sequences (RSS) flanking the variable (V), diverse (D), and joining (J) segments. RSS encompasses a heptamer of seven conserved base pairs (CACAGTG), a spacer region of 12 or 23 variable nucleotides and conserved nanomer (ACAAAAACC). Every V, D, and J segment is flanked with 12-RSS (12 base pair pacer) on one side and with 23-RSS (23 base pair spacer) on the other side. RAG creates single-strand DNA nicks within RSS regions and uses the reactive 3′-OH to disrupt the complementary DNA strand. The segment between 12-RSS and 23-RSS (12/23 rule) is permanently deleted from a chromosome and the ends are covalently joined (Fig. 19.4) [3,62].

While the RSS ends (deleted DNA sequence) are blunt and can be directly ligated, the chromosomal coding ends form hairpin structures that must undergo processing before ligation. The hairpin opening requires the endonuclease activity of Artemis. As expected, deficiency of this nuclease results in accumulation of unopened ends in the maturating B and T cells [35]. Once the coding ends are open, an array of enzymes, such as polymerases and possibly different nucleases act on these ends. A particular role is played by the TdT polymerase, which has a unique ability to attach random nontemplated nucleotides to the overhangs of coding ends. The extensive processing of ends is required to interspace nucleotide sequences, and thus, increase diversity of Ig and TCR [63].

The final ligation of coding ends is highly complex and is exclusively carried out by C-NHEJ machinery. This tunneling function is attributed to RAG proteins, which tether the coding ends and possibly concurrently block the access of other DNA-repair pathways. C-terminal mutation of murine RAG2 permits A-NHEJ to mediate this process [64]. Interestingly, XLF seems to be redundant for successful V(D)J recombination in mice and Xrcc4 in humans [54]. In contrast, knockout or hypomorphic mutations of any other C-NHEJ component results in RS-SCID due to inability to rejoin coding ends of V, D, and J segments in both mice and humans [3,62].

CSR is exclusive to peripheral antigen-stimulated B cells that express IgM and IgD on their surface. In similar fashion to the V(D)J recombination, first formation of DSB is initiated by AID (activation-induced cytidine deaminase). This enzyme deaminates cytidine in S-regions flanking the genes coding for IgM, IgD, IgG, IgE, and IgA. In multistep process, this lesion is converted to a DSB and the unwanted DNA segment is permanently deleted from the chromosome. The segments surrounding the break are brought together resulting in a switch of antibody isotype. In contrast, ligation of these segments is not absolutely dependent on C-NHEJ. Genetically engineered B cells to bypass V(D)J recombination undergo robust CSR in mice deficient in C-NHEJ key components, such as Ku70, LIG4, and Xrcc4. The joining junctions in these animals

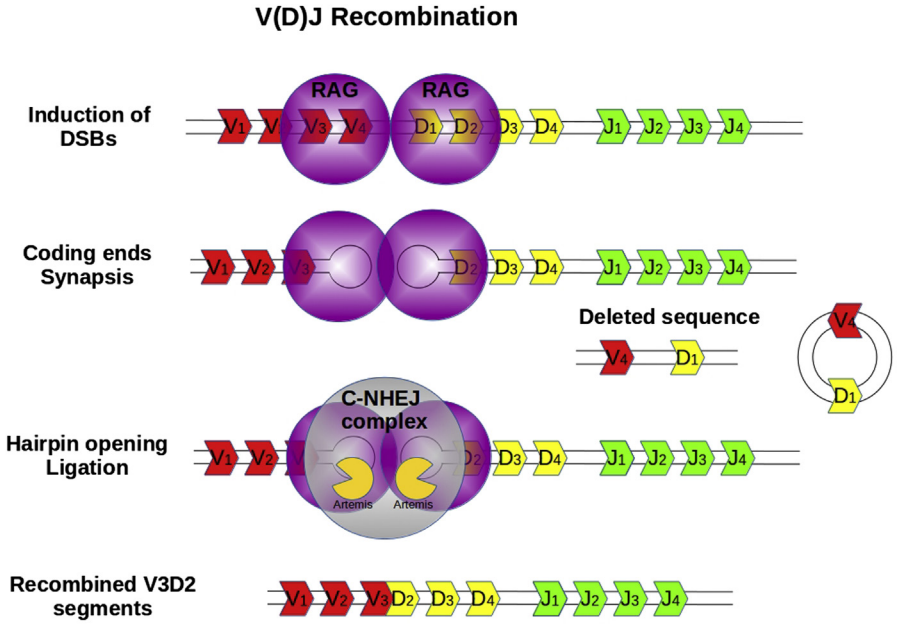

FIGURE 19.4 **Cartoon representing V(D)J recombination.** RAG induces DSBs in RSS regions in the vicinity of segments that are intended to be recombined and tethers the coding ends. The DNA sequence between these segments is permanently deleted from chromosome. The coding ends carry hairpin structure that must be opened before ligation. This is carried out by Artemis. The joining of coding ends is exclusively mediated by C-NHEJ and is tunnelled through RAG.

carry extended microhomology characteristic for A-NHEJ [65–67]. This implies that A-NHEJ has the ability to back up the C-NHEJ machinery in CSR but its physiological role is not understood.

3. ALTERNATIVE NHEJ

While the C-NHEJ has been intensively studied for many years and great knowledge has accumulated, the A-NHEJ, in contrast, has been gaining attention since 2010 as microhomologies (hallmark of A-NHEJ) became linked to chromosomal aberrations in murine and human cells [3,68].

The first evidence for existence of the A-NHEJ came from experiments, in which cells deficient in C-NEHJ component(s) still repair DSB in a template-independent manner [69–71]. Because its activity is mainly seen, when C-NHEJ is inactivated, A-NHEJ is also referred to as backup NHEJ [3,68]. Studies in 2015 further revealed that A-NHEJ may also be a backup mechanism for HR [72,73]. However, it is not clear what is the role of A-NHEJ when the both aforementioned mechanisms are functional. One possibility is, when for whatever reason C-NHEJ and/or HR fails to repair a DSB, then A-NHEJ engages and fixes this break. The fingerprint of A-NHEJ-mediated repair is microhomology with deletions at the repair junctions. This implies that initiation of A-NHEJ requires broken DNA ends to be resected until short homologous overhangs are uncovered (Fig. 19.5). Subsequently, these complementary ends anneal and the break is then directed to A-NHEJ machinery for repair. Due to these short homologies, A-NHEJ has been also designated as microhomology-mediated end joining (MHEJ).

Though the role of microhomology-mediated repair in DNA repair is well established, the nature of this pathway is still heavily disputed. It is not clear whether A-NHEJ is a single pathway or may be distinct pathways. Boboila et al. analyzed CSR junctions from murine B-cells deficient in LIG4 or Ku70 [67]. LIG4-deficient cells showed almost exclusively microhomology-mediated repair of junctions, but in contrast, Ku70-deficient cells carried a substantial fraction of direct junctions with no microhomology. Direct junctions are usually seen in WT cells. Based on these results, Ku70-dependent and Ku70-independent A-NHEJ pathway has been proposed, the latter able to mediate both direct and microhomology-mediated junctions [67]. However, it remains to be deciphered how these differences occur. Since the activity of A-NHEJ is mainly seen in cells deficient in C-NHEJ components, other scientists have suggested that what has been designated as A-NHEJ, is merely C-NHEJ, in which a redundant protein substitutes a missing component, for example, Lig3 substitutes for Lig4 [3,74].

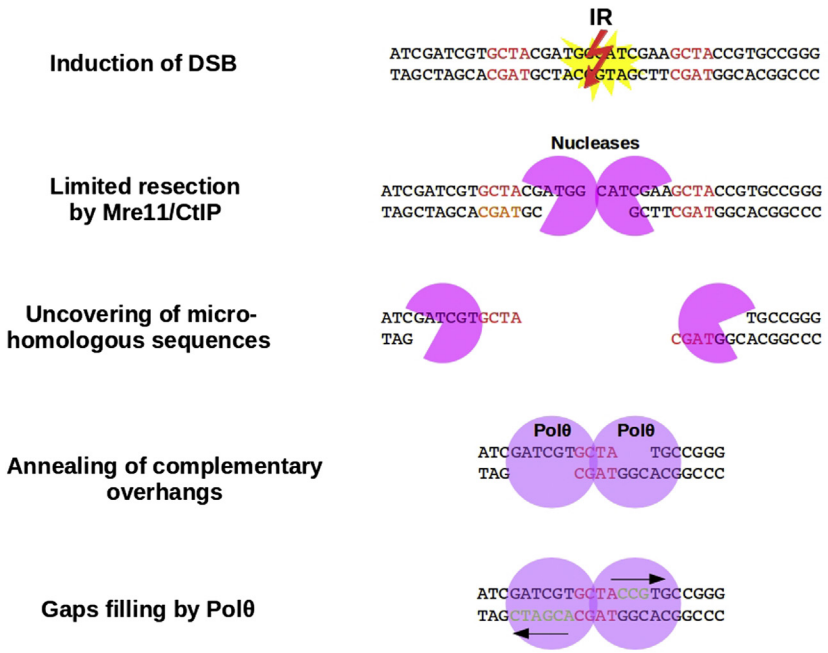

FIGURE 19.5 Cartoon of limited end resection and gap filling. Microhomology is highlighted with *red*. After double-strand break (DSB) induction, incoherent DNA ends undergo limited resection until homologous overhangs are uncovered. Pol θ stabilizes the hybridized overhangs and uses the opposite strand to fill in the gaps. *Arrows* indicate the direction of gap filling.

3.1 Components of A-NHEJ

Based on the literature many different proteins have been implicated to play a role in the A-NHEJ. Some of them are PARP1 (poly-ADP-ribose polymerase 1), Xrcc1, Lig1 and Lig3, Mre11, CtIP (CtBP-interacting protein), and Pol θ (polymerase theta). All of these components and others that are not mentioned here have been already assigned to distinct DNA-repair pathways. For instance, both CtIP and Mre11 have been assigned to HR-mediated repair and PARP1 was first allocated to base excision repair [68]. Thus, it makes it very difficult to define the A-NHEJ pathway.

Although it is not understood how and when cells choose to initiate A-NHEJ over C-NHEJ or HR-mediated DNA repair, there are supporting evidences that PARP1 and the end-processing nuclease CtIP and Mre11 may play a pivotal role in this process. Common step to each DNA-repair pathways is sensing of the break that is mediated by pathway-specific complex (sensor). Simplified, whereas Ku70/80 complex is the DSB sensor for C-NHEJ-mediated repair, MRN complex senses for HR [7,75]. Similarly, PAPR1 may be the sensor protein for A-NHEJ because it is able to bind single- and double-stranded DNA ends and is instantly recruited to DSBs. In addition, inhibition or knockdown of PARP1 counteracts activation of A-NHEJ. PARP1 interaction with broken DNA ends causes its heavy auto-poly-ADP-ribosylation that promotes recruitment of MRN, CtIP, and LIG3/Xrcc1 complex, possibly through direct interaction [76,77].

MRN complex is composed of three proteins Mre11, Rad50, and NBS and avidly binds to DNA DSBs [75]. Based on crystallographic structure, MRN complex tethers broken DNA ends and serves, on the other hand, as a docking platform for CtIP at the break [78,79]. Both Mre11 and CtIP bestow the MRN complex with nuclease activity and carry out initial limited end resection (Fig. 19.5). This resection is restricted to maximum of two to three hundred nucleotides and is needed to uncover microhomologous (few nucleotides long) complementary overhangs. Annealing of these overhangs is possibly required for initiation of A-NHEJ-mediated ligation. However, if cells choose to progress to HR-mediated repair, then there is a switch to an extensive resection over thousand nucleotides mediated by a different set of nucleases, such as EXO1 and BLM [80,81]. At present, it is not understood how the switch/pathway choice is made.

Another unanswered question is how the access of sensor proteins to DSBs is determined. It has been shown that Ku70/80, MRN, and PARP1 compete for binding to broken DNA ends. However, Ku70/80 complex seems to outcompete both MRN and PARP1 making C-NHEJ the default DNA-repair pathway [82–84]. Thus, it is understandable that Ku70 or Ku80 deficiency would favor the binding of PARP1 to a DSB and promote A-NHEJ, but how does deficiency of LIG4 or other C-NHEJ components promote A-NHEJ is not clear [66,67]. One possibility is that in the absence of C-NHEJ components, Ku70/80 binding to DNA ends cannot be stabilized and the complex falls off making the DNA ends again accessible for PARP1 or MRN complex [84,85].

Analog to the role of polymerases (Pol λ and Pol μ) in the C-NHEJ, in 2015, Pol θ was reported to play a role in A-NHEJ. Kent et al. presented in an in vitro assay that, once overhangs with microhomology are annealed, Pol θ stabilizes the hybridized sites of the overhangs and uses the opposing strand to fill in the gaps with complementary nucleotides (Fig. 19.5) [86]. In vivo studies have further supported the role of Pol θ in A-NHEJ and demonstrated its competitive nature with HR. Interestingly, cells compromised in HR due to BRCA1 deficiency rely on A-NHEJ as a backup mechanism and knockdown of Pol θ results in synthetic lethality making it an attractive chemotherapeutic target in a subset of cancers [72,73].

Multiple research groups have shown that the final step of ligation is mostly carried out by LIG3, possibly in complex with its binding partner Xrcc1. Depletion of this ligase in cells or cell extracts significantly reduced A-NHEJ-mediated events [87,88]. Della-Maria et al. provided an interesting observation that in wild-type (WT) cells, MRN and LIG3/Xrcc1 are associated [89]. Upon IR exposure, MRN and LIG3/Xrcc1 dissociate possibly to participate in distinct DNA-repair pathways. In contrast, MRN and LIG3/Xrcc1 remain associated in cells that are deficient in LIG4 or DNA-PKcs, suggesting redirection to A-NHEJ pathway [89]. In line, ablation of nuclear LIG3 decreased translocation rate and remaining translocation did not show bias toward microhomology. Concomitant knockdown of LIG1 but not of LIG4 further suppressed translocation rate making LIG1 the possible backup ligase for LIG3 [90].

The stability of LIG3 is dependent on its binding partner Xrcc1 that is analogous to LIG4/Xrcc4 complex. Ablation of Xrcc1 causes functional deficiency of LIG3 [45]. However, there are convicting studies showing that Xrcc1 may be redundant for A-NHEJ. Knockout of Xrcc1 in WT or in Xrcc4-deficient B cells did not affect A-NHEJ-mediated CSR and IgH/c-myc translocations. This is in line with the work of Soni et al. showing that Xrcc1 is not required for translocations in MEF cells [91]. Based on previous reports that in the absence of LIG3, LIG1 was still driving translocations, these studies further support an important role of LIG1 in A-NHEJ pathway and not necessary just as a backup ligase for LIG3 [65,91].

3.2 Role of A-NHEJ in Chromosomal Aberration

Translocations and other chromosomal aberrations are the hallmark of cancers and can determine the nature of a tumor as well as response to radiochemotherapy. In 2010s, microhomology signature was reported at the breakpoints of translocations

and other chromosomal aberrations providing evidences for A-NHEJ as the executive mechanism for genomic instability under certain circumstances [2,3,92].

Animals deficient in Xrcc4/p53 or LIG4/p53 succumb uniformly from pro-B cell lymphomas that carry oncogenic translocations between chromosome 12 and 15 t(12; 15) resulting in IgH/c-myc fusion [93]. Analogously, non-hematopoietic mouse cells deficient in Ku70, Xrcc4, or LIG4 showed increased rate of translocations. Comparison of breakpoint junctions between WT and C-NHEJ component–deficient cells revealed similar characteristics, such as deletions, insertions, and microhomologies [90,94,95]. In line with the role of A-NHEJ in translocations, depletion of CtIP resulted in decreased translocation rate and reduced microhomologies in WT cells. All these studies support the role of A-NHEJ as a major mediator of translocations in mammalian cells. However, this concept was challenged in 2014 in human cells. Ghezraoui et al. found in multiple human cell lines including HCT116 that deficiency of Xrcc4 or LIG4 decreased translocation rate but remaining translocations carried microhomologies at breakpoint junctions and knocking down CtIP decreases translocation rate [96]. Thus, the authors concluded that C-NEHJ presents the major mechanism of translocation in humans and A-NHEJ plays only marginal role [96]. In contrast, Soni et al. presented opposite results supporting the finding in murine cells. In their hands, translocation rate was increased in the same LIG4-deficient HCT116 cells and frequency of the translocations was decreased by PARP inhibitor [91]. Intermediate results came from analysis of germline chromosomal rearrangement in human patients. Here, 31% breakpoint junctions disclosed microhomology [97]. Thus, eventual species-specific differences between humans and mice remain to be elucidated.

Interestingly, increased A-NHEJ activity has been demonstrated in leukemia cells expressing Bcr-abl or FLT3/ITD (FMS-like tyrosine kinase/internal tandem duplication). Bcr-abl is an oncogenic fusion protein resulting from t(9; 22) translocation and is considered to be the causative mechanism of CML (chronic myelogenic leukemia), whereas FLT/ITD is pathognostic for AML (acute myelogenic leukemia). Cells carrying either of these constitutively active kinases showed impaired DSB repair attributed to deregulation between C- and A-NHEJ pathways [98–100]. However, from these studies it is not clear whether impaired balance between these two mechanisms is a consequence of bcr-abl or FLT3/IDT expression or these aberrations were induced by preexisting increased A-NHEJ activity.

4. END PROCESSING

The nature of DNA-damaging agents determines the complexity of DSBs. Particularly, IR- and ROS-induced damage produce DNA ends that show highly complex structures and are not compatible for direct ligation. Such ends may contain abasic nucleotides, nucleotides missing 3′-OH or 5′-phosphate group, or overhangs with no or only partial complementarity. Ends with hairpin structures arise during V(D)J recombination (Fig. 19.4). Common to all these DNA ends is a requirement for adequate processing before the break can be sealed. On the other hand, coherent or blunt end that can be directly joined needs to be protected from enzymatic trimming [101].

To deal with the complexity of DNA DSBs, an array of regulatory and end-processing components has evolved. Both DNA-PKcs and LIG4 complex have been shown to be recruited to DSBs before the end-processing enzymes and may determine the accessibility to the DNA ends. Central role could be assigned to the ABCDE and PQR phosphorylation clusters of DNA-PKcs that reciprocally regulate end-processing, among others through interaction with Artemis [26,37,48,49]. In line, LIG4 complex serves as docking platform for end-processing enzymes and additionally, LIG4 alone possesses ability to ligate non-cohesive ends containing gaps [44,48,49]. Further, Ku70, a member of the DNA–PK complex, has been also implicated in the end-processing by removing abasic nucleotides [15]. Altogether, it seems that C-NHEJ complex possesses the ability to deal with less-complex breaks. Possibly, failure of C-NHEJ complex to adequately process the ragged ends may contribute to a switch to limited or extensive end resection revealing homologous sequences for A-NHEJ or HR pathway [2].

Another group of specialized enzymes including PNKP (polynucleotide kinase/phosphatase), aprataxin, and APLF (aprataxin and PNKP-like factor) have been implicated in trimming DNA ends during NHEJ. All three enzymes share a common FHA (fork-head associated) domain that mediates their binding to Xrcc4 or Xrcc1 in phosphorylation-dependent manner [102]. PNKP carries a 5′-kinase and 3′-phosphatase activity and is able to restore compatible 5′-phosphate and 3′-OH group at DNA ends [103]. Aprataxin was found to release AMP from the 5′-DNA ends of abortive DNA ligation intermediates, and thus, producing a 5′-phosphate that can undergo religation. Mutations in the gene coding for aprataxin cause neurological disorder, such as ataxia oculomotor apraxia-1, possibly through neuron death due to accumulation of DNA damage [104]. APLF interacts with Ku80 through the central domain and enhances NHEJ, possibly through its endonuclease activity [102].

A special situation arises when partially complementary ends anneal leaving open gaps that need to be filled in. Members of polymerases X family including Pol λ, Pol μ, and TdT (terminal deoxynucleotidyl transferase) have been implicated

in filling in these gaps. Both Pol λ and Pol μ interact with Ku70/80 and LIG4/Xrcc4 complexes, whereas XLF seems to promote their activity. Deficiency of Pol λ and Pol μ during V(D)J recombination resulted in increased deletion of overhangs. Thus, these polymerases possibly contribute to retention of DNA overhangs, but their exact biological role remains to be determined [8]. TdT takes a special role among these polymerases as it is only expressed in hematopoietic B and T cells that undergo V(D)J recombination. During deletion of V, D, and J segments, TdT polymerase adds randomly nucleotides to overhangs of opened coding ends before they are religated. In consequence, the interspaced nucleotides increase the diversity of antigen-specific antibodies and TCRs [63].

5. CONCLUSIONS

Maintenance of genomic stability is of paramount importance for single cells, living organisms, and for conservation of the species. Inability to maintain genome stability has known deleterious consequences, such as cell death, malignant transformation causing cancer, and when germ cells are affected passing of diseases to subsequent generations. On the other hand, cells must accommodate certain level of mutability to ensure evolutionary adaptation. To deal with all these challenges, cells developed DNA-damage response, the ability to sense DNA damage and respond to it in adequate way. This involves several DNA-repair pathways, extensively reviewed in this book as well as activation of signaling pathways that synchronize the cell cycle, DNA replication, and cell metabolism with DNA repair and induce apoptosis, if repair fails.

Nowadays, after decades of intense studies addressing the DNA-repair mechanisms, the main DNA-repair pathways have been possibly discovered. The DNA pathways are tailored to instantly handle any kind of DNA damage at any location in the genome but differ in their fidelity to execute this function. In case of inability to repair the damage, cells are expected to undergo programmed cell death to prevent accumulation of unwanted aberrations and not to pass them to descending population of cells. Nonetheless, it is widely accepted that accumulation of chromosomal aberrations as a consequences of impaired DNA repair leads to malignant transformation, but the causative mechanisms are poorly understood.

A-NHEJ is considered as a backup mechanism of both C-NHEJ and possibly HR. HR is a high-fidelity mechanism that in most cases guarantees 100% faithful repair of DSBs. C-NHEJ, although considered error prone, can directly ligate coherent ends with no change in nucleotides sequence and incoherent ends are processed with minimal nucleotide loss. Cells tolerate well this low level of inaccuracy that may secure certain rate of mutability required to drive evolution. Both C- and A-NHEJ are active through the cell cycle but the latter shows great fluctuations. A-NHEJ peaks in G2 phase and almost vanishes in G1/0 phase of the cell cycle [101]. It is obscure why both C-NHEJ and HR pathway would need to be backed up by low-fidelity mechanism such as A-NHEJ that is blamed to cause genomic instability. It would be understandable that low-fidelity mechanism is activated in differentiated cells that will not commit to enter the cell cycle again such as circulating leukocytes or neurons but in contrast, A-NHEJ reaches its highest activity in G2 phase of dividing cells. May be under physiological conditions, A-NHEJ fulfills a different role and is not mutagenic at all. Merely the conditions, which are chosen to study its function, disclose the wrong nature of this pathway.

Possibly, better characterization of A-NHEJ at the molecular level and better understanding of pathway choice may provide some clarity to the earlier questions. However, the greatest limitation in understanding the complexity of DNA repair and any other cellular processes is attributed to the technics that are at present used in laboratories.

Cellular processes are highly dynamic; billions of molecules classified to hundreds of pathways are functioning at the same time in a single cell and carry out thousands of different reactions. But the vast majority of laboratory techniques enables just to take a snapshot of highly dynamic metabolism in a large number of cells. At present, live-time imaging is in its infancy and allows simultaneous tracing of few molecules at best with low resolution. Studying cellular process with current technics, it is like trying to reproduce and understand the dynamic life of New York City based on static pictures in "google maps." Only development of techniques that allow tracing hundreds or thousands molecules in real time at the resolution of single molecule in a single cell will provide better understanding of all these complex processes. Thus, it is a long way to go until we truly understand the DNA-repair pathways.

GLOSSARY

Chromosomal aberrations All unwanted changes in the genomic DNA.
Heterodimer Complex of two distinct proteins.
Heterotrimer Complex of three distinct proteins.
Microhomology Few nucleotides-long complementary DNA sequences.
Paralog A gene, which arose by duplication and evolved new function.
Processing Removing/adding of nucleotides at free DNA ends.
Radiosensitivity Increased rate of cell death to ionizing radiation.

Sensing To detect a double-strand break.
Synapsis Complex of proteins holding broken DNA ends together.
Tethering Holding two DNA ends together.

LIST OF ABBREVIATIONS

AID Activation-induced cytidine deaminase
APLF Aprataxin and PNKP-like factor
ATM Ataxia telangiectasia related
ATR Ataxia telangiectasia and Rad3 related
Bcr-abl Breakpoint cluster region-abl1 gene
BRCT BRCA1 C terminal
CD Catalytic domain
CSR Class switch recombination
CTD C-terminal domain
CtIP CtBP-interacting protein
DBD DNA-binding domain
DNA-PKcs DNA-protein kinase catalytic subunit
DSBs Double-strand breaks
FHA Fork-head associated
FLT3/ITD FMS-like tyrosine kinase/internal tandem duplication
HEAT Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), yeast kinase TOR1
HR Homologous recombination
Ig Immunoglobulin
IR Ionizing radiation
MRN Mre11, Rad50, NBS
NBS Nijmegen breakage syndrome
NHEJ Nonhomologous end joining
PARP Poly-ADP-ribose polymerase
PIKK Phosphatidyl inositol-3 kinase-like protein kinase
PNKP Polynucleotide kinase/phosphatase
Pol θ Polymerase theta
RAG Recombination-activating gene
ROS Reactive oxygen species
RSS Recombination signal sequence
RS-SCID Radiosensitive severe combined immunodeficiency
SAP SAF-A/B, Acinus, and PIAS motifs
TCR T-cell receptor
TdT Terminal deoxynucleotidyl transferase
vWa von Willebrand A domain
Xrcc4 X-ray repair cross-complementing protein

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