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Repair of DNA Double-Strand Breaks by the Non-homologous End Joining Pathway

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Abstract

DNA double strand breaks (DSBs) pose a serious threat to genome stability. In vertebrates, these breaks are predominantly repaired by non-homologous end joining (NHEJ), which pairs DNA ends in a multi-protein synaptic complex to promote their direct ligation. NHEJ is a highly versatile pathway that utilizes an array of processing enzymes to modify damaged DNA ends and enable their ligation. The mechanisms of end synapsis and end processing have important implications for genome stability. Rapid and stable synapsis is necessary to limit chromosome translocations that result from the mispairing of DNA ends. Furthermore, end processing must be tightly regulated to minimize mutations at the break site. Here we review our current mechanistic understanding of vertebrate NHEJ, with a particular focus on end synapsis and processing.

Keywords

DNA repair; DNA double-strand break; non-homologous end joining; DNA end synapsis; DNA end processing

1. INTRODUCTION

DNA double strand breaks (DSBs) are extremely toxic, and elaborate pathways exist to repair them. Failure to repair even a single DSB can lead to cell death, as persistent DSBs can trigger apoptosis. Moreover, misrepair of breaks can lead to pathological genomic alterations. For example, chromosome translocations, a major driver of oncogenesis, result from improper joining of DSBs from different chromosomes (1). In addition, error-prone polymerases and nucleases can generate localized mutations at the repair junction (2). Thus, DSB repair pathways must be tightly regulated to maximize fidelity.

Two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), are responsible for repairing the majority of DSBs. NHEJ is the predominant pathway in human cells and repairs up to ~80% of all DSBs (3). During NHEJ, DNA ends are brought together by a multi-protein synaptic complex and directly ligated (2).

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In comparison, during HR, 5′→3′ resection at the DSB generates a 3′ single-stranded DNA overhang that invades the sister chromatid, which acts as a template for repair (4, 5). Disruption of either NHEJ or HR dramatically sensitizes cells to DSB-inducing agents and leads to spontaneous chromosomal aberrations, suggesting that the two pathways are complementary (6). The requirement of a sister chromatid in HR means that in most organisms, this repair pathway is restricted to the S and G2 phases of the cell cycle, whereas NHEJ is active throughout the cell cycle.

In addition to NHEJ and HR, the minor pathways of alternative end joining (alt-EJ) and single-strand annealing (SSA) also contribute to DSB repair. Both of these pathways act on resected DNA substrates and therefore compete with HR. Alt-EJ is a mutagenic repair pathway involving the multi-functional polymerase Pol θ that directly ligates partially resected DNA ends together. Alt-EJ relies on microhomologies near the DSB and is characterized by insertions and deletions (7). SSA anneals homologous repetitive sequences flanking a DSB. It requires extensive resection and results in a deletion of the sequence between the annealed repeats (4, 8).

How cells choose among these pathways to repair a given DSB choice is a central question in genome maintenance. Whether or not a DSB undergoes 5′→3′ resection is a critical determinant of pathway choice, as the resulting 3′ ssDNA overhang is a poor substrate for the NHEJ machinery but is required for HR, alt-EJ, and SSA (9, 10). The decision to resect appears to be a dynamic process governed by opposing factors both at the DSB itself (e.g., the anti-resection NHEJ factor Ku vs. the resection initiator MRN) and the surrounding chromatin (e.g., the anti-resection factors 53BP1 and Shieldin vs. pro-resection factor BRCA1) (9, 10). Here, we focus on our current understanding of the NHEJ pathway and direct readers to excellent reviews of other pathways and pathway choice, cited above.

2. AN OVERVIEW OF THE NHEJ PATHWAY

NHEJ proceeds through a set of mechanistically distinct steps to join DNA ends (Figure 1). Upon formation of a DSB, the DNA ends are rapidly bound by the Ku70/80 heterodimer, an extremely abundant ring-shaped molecule that tightly encircles DNA (11). Due to its role as a recruitment hub for many downstream NHEJ factors, Ku binding is a critical DNA end detection step that initiates the assembly of the NHEJ machinery (Table 1). One downstream NHEJ factor is DNA-PKcs, a large protein kinase belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family. DNA-PKcs recognizes DNA-bound Ku, forming the DNA-PK holoenzyme (12). DNA binding stimulates DNA-PKcs kinase activity, leading to the phosphorylation of numerous NHEJ and DNA repair factors (13). Although the functional consequences of many of these phosphorylation events remain unclear, autophosphorylation of DNA-PKcs appears to be critical for DSB repair (Sections 4.3 and 5.4).

Ligation requires close alignment of DNA ends in a synaptic complex (Section 4). Many NHEJ factors have been implicated in synapsis, including the paralogs XRCC4 (14), XRCC4-like factor (XLF) (15, 16) and PAralog of XRCC4 and XLF (PAXX) (17, 18). Ultimately, DNA ligase IV (LIG4), which forms a constitutive complex with XRCC4,

catalyzes ligation (19). Notably, LIG4 can tolerate certain terminal mismatches and damaged bases (20, 21), a unique feature among vertebrate ligases. Nonetheless, many DNA end structures are incompatible for direct ligation. Accordingly, a large number of end processing factors including polymerases and nucleases are recruited to DSBs and act on the ends to prepare them for ligation (2) (Table 1).

Decades of investigation have established the importance of many NHEJ factors and outlined the major steps of the pathway (Figure 1). Current questions in the field include how these factors cooperate with each other at the molecular level, and how the distinct steps of NHEJ are coordinated and regulated. These topics and their implications for genome stability are the focus of this review.

3. PHYSIOLOGICAL ROLES OF NHEJ

NHEJ is responsible for repairing spontaneous DSBs arising from myriad sources, as well as some developmentally programmed DSBs. Given its importance in genome maintenance, misregulation of NHEJ is associated with cancer and other human diseases. Moreover, inhibition of NHEJ has emerged as a means to bolster DSB-inducing cancer therapies or alter genome engineering outcomes. Here, we provide an overview of the various physiological roles of NHEJ.

3.1 Spontaneous DNA double strand breaks

Spontaneous DSBs occur roughly 50 times per day per somatic cell in mammals (22). Major causes of spontaneous DSBs are reactive oxygen species (ROS), a byproduct of aerobic respiration, and environmental ionizing radiation (IR). Both ROS and IR generate single-strand DNA breaks (SSBs) (23), which may become DSBs if there is another SSB nearby on the opposite strand. Such two-ended DSBs are readily repaired by NHEJ. One-ended DSBs are generated when a replication fork encounters an unrepaired SSB (24). In contrast to two-ended DSBs, single-ended DSBs require HR for accurate repair, and repair by NHEJ is associated with toxicity (25). Other sources of DSBs include nuclease activity and abortive topoisomerase activity. Finally, deprotected telomeres are erroneously recognized as DSBs, potentially resulting in NHEJ-mediated chromosome fusions (26).

3.2 The role of NHEJ in V(D)J recombination and class switch recombination

The vast diversity of the vertebrate adaptive immune system is achieved in part through programmed rearrangements of antigen receptor genes. These rearrangements—V(D)J recombination and class switch recombination (CSR)—proceed through DSB intermediates that are ultimately repaired by NHEJ. Here, we provide a brief overview of the involvement of NHEJ in V(D)J recombination and CSR and refer readers to excellent in-depth reviews of these processes (27–31).

V(D)J recombination selects one V, one D, and one J “coding” segment and joins them together to assemble the antigen-binding variable region of the immunoglobulin (Ig) heavy chain and T cell receptor (TCR) β chain (Figure 2A). Ig light and TCR α chains lack D segments and undergo V-to-J rearrangement. Because the germline contains multiple copies of each segment type (e.g., 44 V segments, 23 D segments, and 6 J segments in the human

immunoglobulin heavy chain locus), the many possible gene segment permutations augment immune receptor diversity. V(D)J recombination is initiated by the RAG recombinase, which binds to a pair of short recombination signal sequences (RSSs) that flank V, D, and J coding segments (Figure 2B) (30). RAG introduces DSBs between the RSSs and coding segments, generating blunt "signal ends" and hairpin-capped "coding ends." DNA cleavage by RAG follows the RSS "12/23 rule." RSSs consist of conserved heptamer and nonamer sequences separated by spacers of either 12 or 23 basepairs (12RSS and 23RSS). RAG promotes appropriate V(D)J exon assembly by initiating cleavage only when bound to both a 12RSS and 23RSS (30). For example, at the Ig heavy chain locus (IgH), V, D, and J segments are flanked by 23RSSs, 12RSSs, and 23RSSs, respectively. Thus, the 12/23 rule allows V-to-D joining and D-to-J joining while precluding direct V-to-J joining.

Following DSB induction, RAG channels the DNA ends to the NHEJ pathway, which is strictly required for repair (Figure 2B) (30). NHEJ generates both "signal joints" (ligation of two signal ends) and "coding joints" (ligation of two coding ends). NHEJ directly ligates blunt signal ends, forming "excision circles" that contain the intervening sequence between two coding segments. Hairpin-capped coding ends must be opened by the Artemis nuclease to allow subsequent joining (32). Artemis cleaves one to four nucleotides 3′ of the hairpin tip, generating a palindromic 3′ overhang of variable length. These overhangs then undergo templated extension by the NHEJ-associated polymerases pol λ and pol μ (see Section 5) at Ig heavy and light chain loci, respectively (33, 34), and template-independent extension by TdT (35). In contrast to the relatively uniform and conservative repair of spontaneous DSBs (see Section 5), heterogenous processing by Artemis and the NHEJ polymerases results in coding joint variability that greatly expands the repertoire of antigen binding receptors (29). We will discuss the mechanistic underpinnings of NHEJ fidelity in Section 5.

NHEJ also repairs DSB intermediates programmed during CSR, which rearranges IgH constant (C_H) regions that define immunoglobulin isotype (Figure 2C) (28). Upon B cell activation, DSBs are induced at "switch" regions, and subsequent repair alters which C_H region is juxtaposed with the expressed V(D)J segment. This process replaces the default μ C_H region with γ, ε, or α C_H regions, resulting in an isotype switch from IgM to IgG, IgE, or IgA, respectively. DSB induction at switch regions relies on activation-induced cytidine deaminase (AID) (36), which deaminates cytosine bases to generate uracil, leaving a mismatched U:G basepair. Repair of this lesion by either DNA mismatch repair (MMR) or base excision repair (BER) proceeds through nicked intermediates, and nicks on opposite strands within a few basepairs result in a DSB (31). Although NHEJ is the primary pathway responsible for joining DSBs during CSR, alt-EJ can serve as a back-up pathway (37), unlike in V(D)J recombination.

Productive class switching requires that a DNA end arising from a DSB at the μ switch region (S_μ) be joined to a DNA end arising from a DSB at a downstream acceptor switch region. Unlike V(D)J recombination, CSR does not make use of a sequence-specific recombinase to direct joining of the appropriate gene segments. Instead, CSR appears to rely on transcription and three-dimensional genome architecture (27, 31). Transcription at switch regions exposes single-stranded DNA tracts required for AID activity, thereby ensuring

DSBs are induced at the appropriate locations. Moreover, chromatin looping juxtaposes S_μ and acceptor S regions to promote productive CSR (31).

3.3 NHEJ and human health

Given the critical role in repairing both spontaneous and programmed DSBs, deficiencies in NHEJ have various adverse effects on human health. Mutations in the genes encoding XLF, LIG4, DNA-PKcs, and Artemis confer severe combined immunodeficiency (SCID) as a result of defective V(D)J recombination (reviewed in (38)). A subset of SCID patients also exhibit radiosensitivity (RS-SCID), and human cells with deficiencies in core NHEJ factors are generally hypersensitive to IR. Some SCID patients also present with developmental abnormalities, including microcephaly and/or growth delays. The molecular origins of these developmental abnormalities are less clear but may be caused by neuronal apoptosis, which is observed in NHEJ-deficient LIG4-null mice (39–41).

Whereas inactivation of NHEJ is associated with SCID and radiosensitivity, the misregulation or hyperactivation of NHEJ machinery has been linked to cancer and resistance to cancer therapy. Chromosome translocations, a frequent driver of tumorigenesis, are generated by NHEJ in human cells (42). NHEJ has also been implicated in chromothripsis, a mutational phenomenon in cancer that involves the shattering and rearrangement of a chromosome (43). Moreover, several studies have reported that core NHEJ factors are overexpressed in certain tumor tissues (44), and overly active NHEJ is associated with resistance to DSB-inducing chemotherapy and radiotherapy (45). As such, NHEJ components have emerged as drug targets for cancer therapy (45), and DNA-PKcs inhibitors have entered clinical trials (46).

Many laboratories have also disrupted NHEJ to improve genome editing efficiency (47–50). CRISPR-Cas9 and related technologies introduce sequence-specific DSBs to disrupt or edit a target gene. Such DSBs are primarily repaired by NHEJ, and error-prone NHEJ may result in a frameshift mutation that disrupts the targeted gene. However, precise gene editing typically requires addition of a homologous DNA template and repair of the DSB by HR. Although efforts to inhibit NHEJ have yielded modest improvements, the low efficiency of homology-directed repair (HDR) remains the key barrier to precise genome editing.

4. END SYNAPSIS DURING NHEJ

The NHEJ machinery has no means to determine whether a pair of DNA ends arose from the same DSB. Instead, NHEJ relies on end proximity to direct repair. Upon formation of a chromosomal DSB, the motion of DNA ends is largely constrained (51). However, loss of the NHEJ machinery through depletion of Ku results in a significant increase in the local diffusion of DNA ends (52). Therefore, the NHEJ machinery appears to rapidly and stably synapse DNA ends until they are joined, thereby suppressing chromosome translocations. The following sections describe our emerging understanding of the NHEJ synaptic complex.

4.1 Single-molecule methods to probe end synapsis during repair

Investigators have employed a variety of approaches to determine how DNA ends are synapsed during NHEJ. DNA pulldown experiments have been widely used to identify

factors involved in end synapsis, and structural approaches have elucidated how NHEJ sub-complexes could bridge DNA ends. These studies, discussed in subsequent sections, have implicated all core NHEJ factors in end synapsis. Single molecule methods, described in this section, are beginning to reveal how these factors interact with each other and DNA in a highly dynamic synaptic complex, with transient intermediates and changing composition.

Single-molecule Förster resonance energy transfer (smFRET) is an especially powerful approach to study synapsis because it monitors the distance between paired DNA ends in real time during a physiological repair reaction (53, 54). In these experiments, DNAs are differentially labeled near their ends with donor and acceptor fluorophores. When the DNA ends are close together (i.e. less than 80 angstroms apart), excitation of the donor dye leads to energy transfer to the acceptor dye and acceptor fluorescence. FRET efficiency is highly sensitive to the distance between the two dyes and can report on distance changes as small as a few angstroms. smFRET has been used to observe intermolecular synapsis by tethering donor-labeled DNAs to the surface of a cover slip via a biotin-streptavidin linkage and providing acceptor-labeled DNAs in solution (Figure 3A). Alternatively, a long (~2 kilobase pair) DNA substrate labeled at each end with donor or acceptor dyes can be tethered to the surface at an internal site to observe intramolecular synapsis (Figure 3B). Furthermore, NHEJ factors labeled with distinct fluorophores can be added to the NHEJ reaction in order to correlate the stoichiometry and dynamics of these factors with DNA end joining (Figure 3B) (55).

Nanomanipulation-based single-molecule methods have been used to characterize the stability of the synaptic complex under force (56). These experiments utilize a DNA scaffold of two linear dsDNA molecules, each anchored at one end to either a magnetic bead or a glass surface, and the other end loosely tethered by a dsDNA leash (Figure 3C). The free ends undergo synapsis, and force is applied to the DNA scaffold through the magnetic bead by a standard magnetic tweezers instrument. Rupture of the synaptic complex under force leads to an increase in the measured DNA length. Subsequent relaxation of the force allows for reformation of the synaptic complex and permits multiple rupture cycles to be studied on a single DNA substrate. Additionally, optical tweezers-based approaches have been used to study how sub-complexes of the NHEJ reaction interact with DNA (57).

4.2 End synapsis is maintained by a dynamic multi-protein complex that evolves during repair

Single-molecule imaging from our laboratory and others has shown that the NHEJ synaptic complex is not a static structure but instead evolves during repair (Figure 4). smFRET experiments in *Xenopus* egg extracts showed that DNA ends are initially tethered together in a long-range synaptic complex, in which DNA ends are separated by >80 angstroms (53). This long-range complex requires the core NHEJ factors Ku and DNA-PKcs and only persists for ~ 5 seconds on average. DNA-PKcs kinase activity, along with the factors XRCC4-LIG4 and XLF, are required for the transition to a short-range synaptic complex in which the ends are closely aligned for ligation. Importantly, formation of the short-range complex does not require the catalytic activity of LIG4, indicating that LIG4 plays a structural role in synapsis in addition to its enzymatic role in ligation. In contrast to the long-

range complex, the short-range complex is quite stable and can persist for ~100 seconds or more. Formation of the long-range complex is required to proceed to the short-range complex, demonstrating that a structural remodeling of the NHEJ machinery occurs during repair.

Single-molecule magnetic tweezers experiments using a reconstitution of human NHEJ proteins provided further evidence that distinct synaptic complexes form during DSB repair (56). While these studies lacked the spatial resolution to directly measure small distance changes between DNA ends, two synaptic complexes were identified that showed similar stabilities and compositions to the long-range and short-range complexes described above. Ku and DNA-PKcs alone supported fleeting synapsis of DNA ends, with a half-life of ~100 ms. Addition of PAXX dramatically increased the lifetime of this synaptic complex to ~2 seconds, similar to that of the long-range complex observed in *Xenopus* egg extracts. Thus, PAXX (which is also present in egg extracts) and the DNA-PK holoenzyme appear to mediate long-range synapsis. Inclusion of XLF and XRCC4-LIG4 further increased the lifetime of the synaptic complex to ~60 seconds, consistent with the short-range complex observed in *Xenopus* egg extracts. Omission of any one of these factors resulted in a substantial drop in the stability of synapsis and the frequency of end joining. Other single-molecule studies involving reconstitutions of human NHEJ proteins similarly show that end synapsis is a dynamic process and that persistent and close alignment of DNA ends requires XLF and XRCC4-LIG4, although they challenge the requirement of DNA-PKcs in end synapsis (54, 58), a topic that will be described in detail below (Section 4.3).

4.3 Catalytic and non-catalytic roles of DNA-PKcs in end synapsis and joining

DNA-PKcs is a core NHEJ factor whose deficiency severely disrupts NHEJ. Loss of DNA-PKcs or the inhibition of its kinase activity with selective, small-molecule inhibitors sensitizes human cells to DSB inducing agents (59, 60) and blocks end joining in cell extracts (53, 61). DNA-PKcs inhibition also disrupts repair of Cas9-generated breaks by NHEJ (50), and mice expressing catalytically inactive DNA-PKcs display an embryonic lethal phenotype similar to that of *LIG4*$^{-/-}$ and *Xrcc4*$^{-/-}$ mice (62). Beyond its role in NHEJ, DNA-PKcs contributes to telomere maintenance, ribosomal RNA processing, and mitosis (13, 63, 64).

DNA-PKcs is rapidly recruited to DSBs in cells (65). This recruitment occurs through an interaction with Ku that depends, in part, on a flexible C-terminal extension of Ku80 (12, 66). Formation of the DNA-PK holoenzyme activates its kinase activity (12). DNA-PK typically phosphorylates a serine or threonine followed by a glutamine, although this sequence preference is not strict (13). DNA-PKcs phosphorylates many NHEJ and DSB repair factors, but DNA-PKcs itself may be the most critical substrate for NHEJ. DNA-PKcs is autophosphorylated at numerous sites including two distinct clusters known as ABCDE (residues 2609–2647) and PQR (residues 2023–2056). Individual phosphoablating mutations within these clusters have at most a modest effect on sensitivity to DSBs. However, combining mutations, particularly within the ABCDE cluster, leads to profound sensitization to DSB inducing agents in cells (67, 68). We further discuss the roles of DNA-PKcs autophosphorylation in Section 5.4.

Multiple lines of evidence indicate that DNA-PKcs also plays a critical role in end synapsis. DNA-PKcs greatly stimulates the ability of Ku to bridge DNA ends in biochemical assays (69). Consistent with this biochemical activity, cryo-EM and x-ray structures suggest that the N-terminal region of the DNA-PKcs self-associates to bridge DNA ends (70, 71). In addition, depletion of DNA-PKcs from human cell and *Xenopus* egg extracts abolished DNA end synapsis (53, 72). As DNA-PKcs autophosphorylation is believed to occur largely in trans (73), this suggests that DNA-PKcs is likely a component of the synaptic complex. However, other observations suggest DNA-PKcs is not universally essential for NHEJ. Reconstitutions of human NHEJ proteins have provided conflicting results, with some studies concluding that DNA-PKcs is critical for synapsis and end joining (56) and others suggesting that it is dispensable (58). These discrepancies may highlight the difficulty of faithfully recapitulating physiological DNA repair using purified components. Still, IR sensitivity of cells lacking DNA-PKcs is less pronounced than in cells lacking XRCC4 (62). It is therefore possible that, compared to DSB substrates in biochemical experiments, chromosomal DSBs have less stringent requirements for synapsis in certain cellular contexts. For example, chromosomal breaks may be more easily synapsed because they are limited in mobility by the large size of chromosomes and higher order chromatin structure. Nonetheless, the bulk of the evidence across a number of model systems suggests that DNA-PKcs and its kinase activity are broadly important for end synapsis and joining. Future studies will be necessary to dissect the specific contributions of DNA-PKcs at each stage of the repair reaction.

4.4 XLF contributes to the close alignment of DNA ends

XLF was first linked to NHEJ when human patients exhibiting immunodeficiency, radiosensitivity and developmental abnormalities were found to carry mutations in XLF (15, 16). In cultured human cells, loss of XLF substantially attenuates NHEJ and V(D)J recombination (16). XLF is a paralog of XRCC4, and although it has little sequence conservation with XRCC4, it is structurally quite similar (74). Both XLF and XRCC4 exist as homodimers with a globular N-terminal head domain, a coiled-coil domain that mediates dimerization, and an intrinsically disordered C-terminal tail (74–76). XLF and XRCC4 interact through their head domains, and disrupting this interaction severely attenuates NHEJ (55, 77). The C-terminal tail of XLF plays a dual role in end synapsis. First, the extreme C-terminus contains a Ku binding motif (KBM), a short peptide that recruits XLF to DSBs (78). Removing the KBM disrupts end joining in both biochemical and cell-based experiments (79, 80). Second, while the KBM anchors XLF to Ku, the flexible tail allows XLF to explore the NHEJ complex and form interactions with XRCC4 that are required for synapsis. XLF mutants with a shortened tail but intact KBM are deficient in end joining in both *Xenopus* egg extracts and in mouse embryonic stem cells (80). This deficiency is not attributable to known and putative phosphorylation sites in the tail, which are dispensable for joining (81, 82). Furthermore, XLF variants with shuffled tail sequences are competent for NHEJ (80). Thus, the length and likely the flexibility of the XLF tail, rather than its specific sequence, are critical for efficient joining. In support of this model, the C-terminal tail of XLF has poor sequence conservation outside of the KBM but is highly conserved in its length. Because asymmetric XLF mutants containing only a single KBM are proficient in end joining (80), it is unlikely that long XLF tails are required to interact with Ku molecules

on both DNA ends. Rather, XLF variants with internally truncated tails are efficiently recruited to Ku but fail to stabilize LIG4-XRCC4 at DSBs (80), supporting the idea that sufficient XLF tail length appears to allow interaction with XRCC4.

Numerous studies have implicated XLF in DNA end synapsis. Alternating filaments of XLF and XRCC4 have been proposed to be a key structure that mediates synapsis. These filaments have been observed in structural studies (83, 84) and can bridge DNA ends in vitro (75). In superresolution imaging of fixed cells, XLF and XRCC4 puncta have been interpreted as continuous XLF-XRCC4 filaments (54). However, it is unclear how these filaments could allow LIG4 or other end processing factors access to DNA ends and how the interior channel could accommodate DNA-PKcs. By directly imaging fluorescently labeled XLF in *Xenopus* egg extracts, we showed that, while *Xenopus* XLF and XRCC4 can form filaments in vitro, only a single XLF dimer is present within the short-range complex during end joining (55). Short-range complex formation requires the interaction between XLF and XRCC4, and asymmetric XLF mutants that are deficient in XRCC4-interaction in only one monomer were also severely deficient in end synapsis and joining. These results support a model in which a complex of XLF interacting with XRCC4-LIG4 through each of its head domains is necessary to closely align DNA ends. This ternary complex is consistent with structural work suggesting that LIG4 may prevent extensive XLF-XRCC4 filaments (85). Subsequent work in a reconstitution of human NHEJ factors found that end synapsis is not particularly sensitive to XLF concentration, further suggesting that filament formation is not necessary for end synapsis (58). Collectively, these biochemical studies may explain genetic observations that mutations (e.g. XLF L115A) that ablate filament formation in vitro (75, 86), but do not significantly attenuate the XLF–XRCC4 interaction (55), also do not affect end joining in cells (86). Therefore, we propose that a stoichiometric complex of a single XLF dimer interacting with two copies of XRCC4-LIG4 is likely the physiological complex essential for mediating synapsis.

4.5 Ligase 4 is an essential structural factor in DNA end synapsis

LIG4 is a multi-domain enzyme comprised of an N-terminal DNA binding domain, catalytic and OB domains and C-terminal tandem BRCT domains. The region between the two BRCT domains interacts with XRCC4 (87) while the first BRCT domain interacts with Ku (88). In cells, LIG4 interaction with Ku targets XRCC4-LIG4 to DSBs and dramatically stimulates end joining activity (89). As a result, loss of Ku results in a total loss of NHEJ (79, 90, 91).

In addition to its catalytic role in ligation, accumulating evidence implicates LIG4 in DNA end synapsis. LIG4 is required for stable synapsis in both human cell and *Xenopus* egg extracts and in reconstitutions of human NHEJ factors (53, 56, 72). Importantly, the catalytic activity of LIG4 is not required for synapsis. Thus, LIG4 structurally participates in a network of intermolecular interactions that hold DNA ends together. How LIG4 facilitates DNA end synapsis during NHEJ remains unclear. LIG4 engagement of both DNA ends may be necessary to closely align DNA ends (53, 58). Consistent with this possibility, a LIG4-specific structural motif, insert1, facilitates LIG4 engagement of ends with diverse structures (92), and DNA ends are poised to be ligated within the short-range complex (53). Alternatively, before it engages DNA ends, LIG4 may act as an essential “connector” within

the synaptic complex through recruitment of XRCC4. Once present at DNA ends, XLF and XRCC4-LIG4 could form a protein "bridge" that spans DNA ends. These models need not be mutually exclusive. Indeed, a number of distinct connections across the DNA break likely stabilize the synaptic complex and provide it with the flexibility necessary to carry out the diverse set of enzymatic activities required to join complex DNA end structures.

4.6 NHEJ accessory factors and their potential role in end synapsis

Chromatin isolation and mass spectrometry experiments have shown that along with the core NHEJ factors, many "accessory" proteins are recruited to DSBs (93, 94)(Table 1). In contrast to core NHEJ factors, deficiency in accessory factors is characterized by mild impairment of cellular NHEJ. The accessory factor PAXX was identified as an interactor of Ku and a structural homolog of XRCC4 and XLF (17, 18). Accumulating evidence suggests that PAXX is functionally redundant with XLF in certain cellular contexts. The loss of both XLF and PAXX is embryonically lethal in the mouse (95) and leads to profound defects in V(D)J recombination and the response to ionizing radiation in murine G1-arrested pro-B-cells (96). Aprataxin and PNKP-Like Factor (APLF) is an accessory factor recruited to DSBs through its interaction with Ku, XRCC4, and poly (ADP-ribose). Although it possesses nuclease activity in vitro (97), its predominant role in NHEJ appears to be stimulation of repair via stabilization of XRCC4-LIG4 and XLF at DSBs (98). The Werner syndrome helicase (WRN), another accessory factor, is a RecQ-like helicase with $3' \rightarrow 5'$ exonuclease activity. WRN interacts tightly with Ku, which stimulates WRN exonuclease activity in vitro (99) and is required for optimal repair kinetics in cells (100). WRN has also been implicated in DSB repair pathway choice due to its role in suppressing DNA end resection (101). Finally, the modulator of retrovirus infection (MRI, also known as CYREN) interacts with Ku and plays a complex role in regulating NHEJ during the cell cycle. MRI suppresses NHEJ on deprotected telomeres in the S and G2 phases of the cell cycle (102) yet is required for efficient NHEJ in other contexts, including resistance to ionizing radiation and for cells lacking XLF(103).

Each of these accessory factors possesses at least one KBM for recruitment to DSBs. It remains unclear how all of these factors compete for a limited number of Ku binding sites and if this competition impacts the assembly of the NHEJ synaptic complex (104, 105). Three distinct KBMs have been identified that interact with unique sites on Ku. The X-KBM from XLF and the A-KBM motif from APLF interact with Ku80 (98, 100), whereas the P-KBM of PAXX interacts with Ku70 (106). The A-KBM binds to a hydrophobic cleft near the periphery of the von Willebrand factor A-like (vWA) domain of Ku80 (107). Surprisingly, the X-KBM binds the same vWA domain at a site that is buried in the absence of the X-KBM peptide. Thus, a large outward rotation of the vWA domain is required to expose the XLF binding site. It is unknown whether this conformational transition in Ku is regulated by other NHEJ factors. The Ku binding site of the P-KBM remains poorly defined. Biochemical evidence of a ternary complex of Ku-XLF and PAXX on DNA demonstrates that the binding of PAXX and XLF to Ku is not mutually exclusive (106). Furthermore, PAXX binds to the Ku heterodimer and the Ku70 homodimer with similar affinities, suggesting that binding is mediated through Ku70.

In most cases the mechanistic role of accessory factors in NHEJ remains unclear. One possibility is that they influence assembly of the synaptic complex by excluding or stabilizing other NHEJ factors. In the former case, accessory factors may compete with each other and core NHEJ factors for Ku binding. For example, structural studies have shown that the A-KBMs from APLF, WRN, and MRI all bind the same site (108). Therefore, competitive binding by mass action could result in substantial heterogeneity among individual NHEJ synaptic complexes. Alternatively, regulatory mechanisms such as phosphorylation by DNA-PKcs could modulate Ku-KBM affinity and direct an ordered progression of synaptic complex composition. Accessory proteins may also act to stabilize other factors at DNA ends. APLF has been proposed to act as a scaffold protein that recruits a number of DSB repair factors to breaks including LIG4 (98). Similarly, PAXX has been implicated in recruiting and stabilizing the NHEJ polymerase Pol λ at DNA ends (109).

NHEJ accessory factors may also directly contribute to DNA end synapsis. Single-molecule nanomanipulation experiments demonstrated that PAXX increases the stability of the DNA-PK synaptic complex by ~20-fold, although the mechanistic origin of this effect remains unknown (56). Notably, some accessory proteins have multiple KBMs that could span the DNA break and therefore directly synapse DNA ends. Such proteins include MRI and WRN, which contain different types of KBMs at their N- and C-termini. MRI has an A-KBM and an X-KBM at its N- and C-termini, respectively. However, the C-terminal X-KBM fails to bind Ku on its own (100) and may instead play a role in recruiting other DSB repair proteins (103). WRN possesses an N-terminal A-KBM and C-terminal A- and X-KBMs (100). Whether WRN simultaneously utilizes all of these interaction sites within the synaptic complex is currently unknown. However, the vast number of potential interactions between core and accessory factors undoubtedly contributes to the stability of the NHEJ synaptic complex.

In addition to protein factors, noncoding RNAs (ncRNAs) may also contribute to DNA end synapsis during NHEJ. The long ncRNA LINP1 interacts with Ku80 and acts as a scaffold to stabilize Ku and DNA-PKcs at DSBs (110). Moreover, ncRNAs contribute to DSB repair by modulating the activity of p53, sequestering regulators of DSB repair and altering the expression of DNA repair proteins (111). Uncovering additional ncRNAs that influence NHEJ and articulating their mechanism in synaptic complex formation and stability will be important areas of future investigation.

5. THE REGULATION OF ERROR PRONE END PROCESSING

DSB induction frequently yields damaged DNA ends that are not suitable for immediate ligation by LIG4. As such, NHEJ employs a variety of end-processing enzymes that modify DNA ends until they are compatible for ligation (Table 1). NHEJ end-processing enzymes include polymerases and nucleases, which can result in insertions and/or deletions (indels) near the DSB site, and “damage correction” enzymes, which prepare termini for ligation but do not alter sequence information. Although end processing endows NHEJ with important flexibility, it is also potentially mutagenic. In this section, we provide a brief overview of NHEJ end-processing enzymes and focus on how they are regulated to maintain versatility while minimizing mutagenicity.

5.1 Overview of end-processing enzymes and their cognate DNA substrates

The nuclease most clearly implicated in NHEJ is Artemis, which has defined interactions with both LIG4 and DNA-PKcs (112, 113). During V(D)J recombination, Artemis cleaves hairpin intermediates generated by the RAG recombinase at coding ends (Figure 2B, Figure 5), thereby allowing their subsequent joining (32). Artemis-deficient cells are unable to repair a subset of DSBs induced by ionizing radiation, indicating that Artemis also contributes to the repair of some spontaneous DSBs (114). It is unclear which non-hairpin DNA end structures Artemis resolves *in vivo*, but it may remove 5′-aldehyde structures formed by certain radiomimetics (115). Furthermore, the purified protein cleaves various overhang and flap structures *in vitro* (116). APLF, WRN, and primate-specific SETMAR (Metnase) have also been proposed as NHEJ-associated nucleases (117–119). In addition, each of these proteins has non-nuclease activities (Section 4.6) (120–122), and their contributions as nucleases during physiological NHEJ are unclear.

The NHEJ polymerases pol λ, pol μ, and terminal deoxynucleotidyl-transferase (TdT) belong to the X family of DNA polymerases. Each is recruited to DSBs via an N-terminal BRCT domain that allows interaction with Ku and XRCC4-LIG4 (123, 124). Pol λ and pol μ are expressed ubiquitously, whereas TdT is expressed only in developing lymphocytes, where it is utilized during V(D)J recombination (125). The other X family polymerase, pol β, functions primarily in base excision repair, with limited proposed roles in NHEJ (126). The NHEJ polymerases are biochemically specialized for particular DNA end structures: pol λ acts preferentially on 3′ termini that are base-paired to the template strand, whereas pol μ does not require a paired 3′ terminus for templated synthesis (Figure 5) (127). Loss of either pol λ or pol μ alone has distinct effects on repair fidelity, suggesting that polymerase specialization occurs during cellular NHEJ (128). TdT incorporates deoxynucleotides at 3′ termini in a template-independent fashion and contributes to junctional diversification during V(D)J recombination (Figure 5) (29). Surprisingly, pol μ and TdT primarily incorporate ribonucleotides when they are engaged during NHEJ (129). This activity affords NHEJ additional flexibility and likely highlights the evolutionary imperative for rapid DSB repair.

NHEJ utilizes several “damage correction” enzymes that resolve specific chemical blocks to enable ligation without altering sequence information (Figure 5). The bifunctional polynucleotide kinase 3′-phosphatase (PNKP), recruited to DSBs via interaction with XRCC4, phosphorylates 5′-hydroxyl termini and removes 3′-phosphates generated by IR (130). Aprataxin, which also interacts with XRCC4 (131), reverses damage caused by abortive ligation (132). Ligation reactions involve transfer of an AMP moiety to the DNA 5′-phosphate terminus, and this intermediate persists when damaged DNA ends cannot be aligned to complete ligation (133). Aprataxin removes AMP to regenerate the 5′-phosphate terminus for subsequent ligation attempts. Similarly, tyrosyl-DNA phosphodiesterases 1 and 2 (TDP1 and TDP2) regenerate ligatable termini after abortive topoisomerase activity. Topoisomerases cleave and re-ligate DNA to relieve topological stress, proceeding through a phosphotyrosine intermediate that covalently crosslinks the protein to DNA (134). Topoisomerases can stall in the presence of topoisomerase inhibitors or when they cleave within distorted DNA, thereby leaving a covalent block to re-ligation (135). Thus, TDP1 hydrolyzes 3′-phosphotyrosine topoisomerase I intermediates (136) and TDP2 reverses 5′-

phosphotyrosine topoisomerase II intermediates (137) to allow re-ligation by NHEJ or other pathways. In addition, TDP1 has been implicated in removal of diverse 3′-phosphodiester adducts (138), including 3′-phosphoglycolates, which commonly arise at DSBs induced by ionizing radiation (139). Finally, beyond its role as a DSB sensor and recruitment hub, Ku acts as a 5′-deoxyribose-5-phosphate/apurinic-apyrimidinic (5′dRP/AP) lyase near DNA ends (140). This activity removes abasic sites—generated by IR or as intermediates during CSR, among other sources—that would otherwise block repair by NHEJ.

5.2 Coordination of end processing and end synapsis minimizes NHEJ errors

Accumulating evidence indicates that NHEJ employs end-processing enzymes only when necessary and repairs DSBs with minimal indels. Compatible (i.e., blunt or sticky) ends are typically joined by NHEJ without errors in mammalian cells (141–143), human cell extracts (61, 144), and *Xenopus* egg extracts (145, 146). Furthermore, incompatible ends are typically processed as conservatively as possible in the minimum number of steps (128, 146, 147). For example, 5′-hydroxyl ends in principle could be converted to 5′-phosphate ends through either direct phosphorylation by PNKP or nuclease activity, but the former is strongly preferred (146). Thus, end processing during NHEJ appears to be a carefully regulated process that has evolved to minimize errors during DSB repair.

How end processing is regulated directly impacts the fidelity of genome maintenance and the outcomes of genome engineering techniques that introduce DSBs (e.g., CRISPR-Cas9). Indeed, a deeper understanding of end processing regulation could inform efforts to modulate the fidelity of CRISPR-Cas9-generated DSBs. One model of end processing regulation, based on biochemical reconstitutions of purified proteins, posits that each DNA end is processed independently, with modification occurring stochastically and iteratively by a variety of processing enzymes until the ends become compatible for ligation (124, 148). It is unclear, however, how such a model can account for the conservative properties of NHEJ outlined above. Recent work from our laboratory in *Xenopus* egg extracts has elucidated how end processing is coordinated with end synapsis (Section 4.2) and provides a model for how NHEJ minimizes errors (146) (Figure 6). This model comprises three features that promote conservative end joining: first, end processing is blocked by Ku and DNA-PKcs (see section 5.4) until DNA ends enter the short-range synaptic complex. Second, because DNA ends are poised for ligation in the short-range complex, compatible ends are ligated immediately without processing, as observed in multiple experimental systems (61, 141–146). Third, end processing occurs directly within the short-range synaptic complex, which ensures that incompatible DNA ends are rapidly ligated after undergoing the minimal number of processing steps required for compatibility. In support of this idea, perturbations of short-range synapsis (LIG4-XRCC4 deficiency, XLF deficiency, DNA-PKcs inhibition) block a range of end processing activities in human cells, human cell extracts, and *Xenopus* egg extracts (146, 147, 149–152). Moreover, single-molecule FRET experiments that simultaneously monitor end synapsis and end processing in real time directly demonstrate that pol λ and Tdp1 act preferentially in the short-range synaptic complex (146). It is likely that other end processing enzymes exhibit a similar preference. Thus, end processing in the short-range synaptic complex could promote repair with minimal errors without sacrificing versatility.

End processing in the short-range synaptic complex likely requires that LIG4 and processing enzymes alternate in engaging incompatible DNA ends. Although the precise molecular choreography remains unclear, LIG4 may disengage DNA ends to allow processing yet remain associated with the NHEJ complex through its interaction with XRCC4 (153), thereby allowing rapid ligation of newly compatible ends. Moreover, LIG4 engagement of incompatible ends may result in an unproductive “open” conformation or other remodeling of the synaptic complex that facilitates end processing factor recruitment (92, 143, 154). Further biochemical and structural studies are needed to address these possibilities. Finally, an interesting but unresolved question is how end processing is regulated during V(D)J recombination, which is characterized by higher junctional diversity than that observed for non-programmed breaks (29). Unique features of V(D)J recombination, including the involvement of TdT and contributions by RAG, may account for these differences.

5.3 A hierarchical model of end processing

The processing enzymes, if any, that act on a given pair of DNA ends determine the fidelity of NHEJ. Accumulating evidence suggests a model in which end-modifying enzymes are employed hierarchically as an additional layer of regulation to maximize fidelity (Figure 6) (146, 155, 156). Under this model, direct ligation by LIG4 is given highest priority, followed by conservative damage correction activity, and finally sequence-altering polymerase and nuclease activity. Consistent with this idea and as noted above, compatible ends are typically directly ligated without processing. Moreover, LIG4 has the unique ability to join ends with mild nucleotide mismatches or oxidative damage (e.g., 8-oxoguanine), thereby acting as a “translesion ligase” (20, 21, 143). If LIG4 is unable to repair the DSB, conservative damage correction enzymes are prioritized over sequence altering events. For example, ends requiring only 5′-phosphorylation by PNKP for ligation are usually joined without insertions or deletions, and Tdp1 activity is preferred over nuclease activity for the removal of 3′-phosphodiester adducts (146). Among sequence-altering enzymes, gap-filling polymerase activity appears to be prioritized over nuclease activity. In mammalian cells and *Xenopus* egg extracts, non- or partially-complementary ends that could in principle be resolved by either polymerase or nuclease activity are typically subjected only to polymerase activity (143, 146, 157, 158). Gap-filling limited by the availability of single-stranded template may allow for greater preservation of genetic information than heterogeneous nuclease activity. Overall, hierarchical organization of processing enzymes appears to play an important role in restraining the potential mutagenicity of NHEJ.

The molecular mechanisms enforcing such a hierarchy remain unclear but may include the relative abundance of processing enzymes and their affinity for the NHEJ complex. The extent of DNA end accessibility may also regulate processing hierarchy. For example, damage correction enzymes PNKP and TDP1 make minimal contacts with DNA substrates outside the extreme terminus, whereas pol λ and pol μ make more extensive DNA contacts. Thus, a gradual loosening of end protection, perhaps mediated by multiple DNA-PKcs phosphorylation events (see next section), could allow some conservative enzymes to access DNA ends before polymerases. Moreover, the availability of appropriate DNA end chemistry may also contribute to hierarchical end processing. For example, pol λ and pol μ must extend from an undamaged 3′-hydroxyl terminus and are stimulated by the presence of

a 5′-phosphate on the downstream DNA end (159). Thus, efficient polymerase activity may depend on prior preparation of termini by conservative damage correction enzymes.

5.4 Ku and DNA-PKcs are regulators of DNA end accessibility

Protection of DNA ends from unrestricted modification provides another layer of end processing regulation. Initial Ku binding to DNA ends blocks both long-range resection, which would disfavor NHEJ and promote homologous recombination or alternative end joining (160), as well as premature processing by NHEJ enzymes (146). It is unclear, however, how this initial Ku block is alleviated to allow regulated processing in later stages of the NHEJ reaction. An interesting possibility suggested by footprinting studies is that the Ku ring slides inward upon DNA-PKcs association, thereby exposing the DNA end (161). Linking Ku deprotection to association of DNA-PKcs could limit end processing prior to the formation of a synaptic complex. Posttranslational modification of Ku by phosphorylation (162) or ubiquitination (163–168) may also regulate end accessibility.

DNA-PKcs has also long been implicated in regulating DNA end accessibility (reviewed in (169)). Initial association of DNA-PKcs contributes to the protection of ends from aberrant processing (146, 170). Subsequent phosphorylation of DNA-PKcs at two distinct clusters of residues (Section 4.3) has been reported to have opposing effects on end accessibility. Sequence analysis of V(D)J coding joints and repaired I-SceI-induced DSBs in cells expressing mutant DNA-PKcs reveals that phosphorylation at the ABCDE cluster promotes end processing, whereas phosphorylation at the PQR cluster restricts end processing (67, 68, 171). Phosphorylation of DNA-PKcs induces its dissociation from DNA ends (172). Notably, a DNA-PKcs mutant with alanine substitutions at both the ABCDE and PQR clusters shows no defect in dissociation from DNA ends (173). Thus, it appears that phosphorylation of the ABCDE and PQR clusters modulate the interaction of DNA-PKcs with DNA ends independently of complete dissociation, which is mediated by phosphorylation of other unidentified sites. DNA-PKcs autophosphorylation in trans (73) links regulation of end accessibility to synapsis. Further biochemical and structural studies are required to elucidate the molecular details of how phosphorylation of the ABCDE and PQR clusters, as well as of dissociation-inducing sites, regulates access to DNA ends.

The extent to which other NHEJ factors contribute to DNA end protection is unclear. Extensive XLF-XRCC4 filaments have been proposed to protect DNA ends (174), but such filaments are not likely to form during physiological NHEJ (55). An intriguing possibility is that LIG4 directly engages DNA ends, thereby prohibiting access by processing enzymes before ligation has been attempted (Figure 6). To address this possibility, it will be important to determine whether engagement of a given DNA end by LIG4 and other end-modifying enzymes is mutually exclusive.

6 Conclusion and outlook

Here, we have reviewed how DNA end synapsis and regulated end processing during NHEJ promote efficient and faithful DSB repair. Synapsis is carried out by a dynamic multi-protein complex that evolves during repair. DNA ends are initially weakly tethered together in a long-range complex that depends on the core NHEJ factors Ku and DNA-PKcs

and is stabilized by the accessory factor PAXX. Close and stable alignment of DNA ends in a short-range complex requires XLF and XRCC4-LIG4 along with the kinase activity of DNA-PKcs. Future work is necessary to determine the full complement of molecular interactions required for end synapsis. Outstanding issues include the precise structural role of LIG4 in short-range synapsis; whether transition from the long-range to short-range synaptic complexes is due to changes in protein composition or instead a structural remodeling of pre-bound factors; and the roles of NHEJ accessory factors in the assembly and stability of the synaptic complex.

End processing during NHEJ is finely tuned to minimize mutagenesis. DNA ends are initially protected from processing, which is limited to the ligation-poised short-range synaptic complex. This mechanism prioritizes ligation over end processing and promotes rapid ligation after the minimum number of processing events. In addition, processing appears to occur hierarchically, with conservative end processing activities prioritized over mutagenic ones. Unanswered questions in the regulation of end processing include how end protection is relieved to allow processing and ligation, and how hierarchical processing is enforced. Answers to these and other mechanistic questions will be important to fully understand how NHEJ contributes to genome maintenance.

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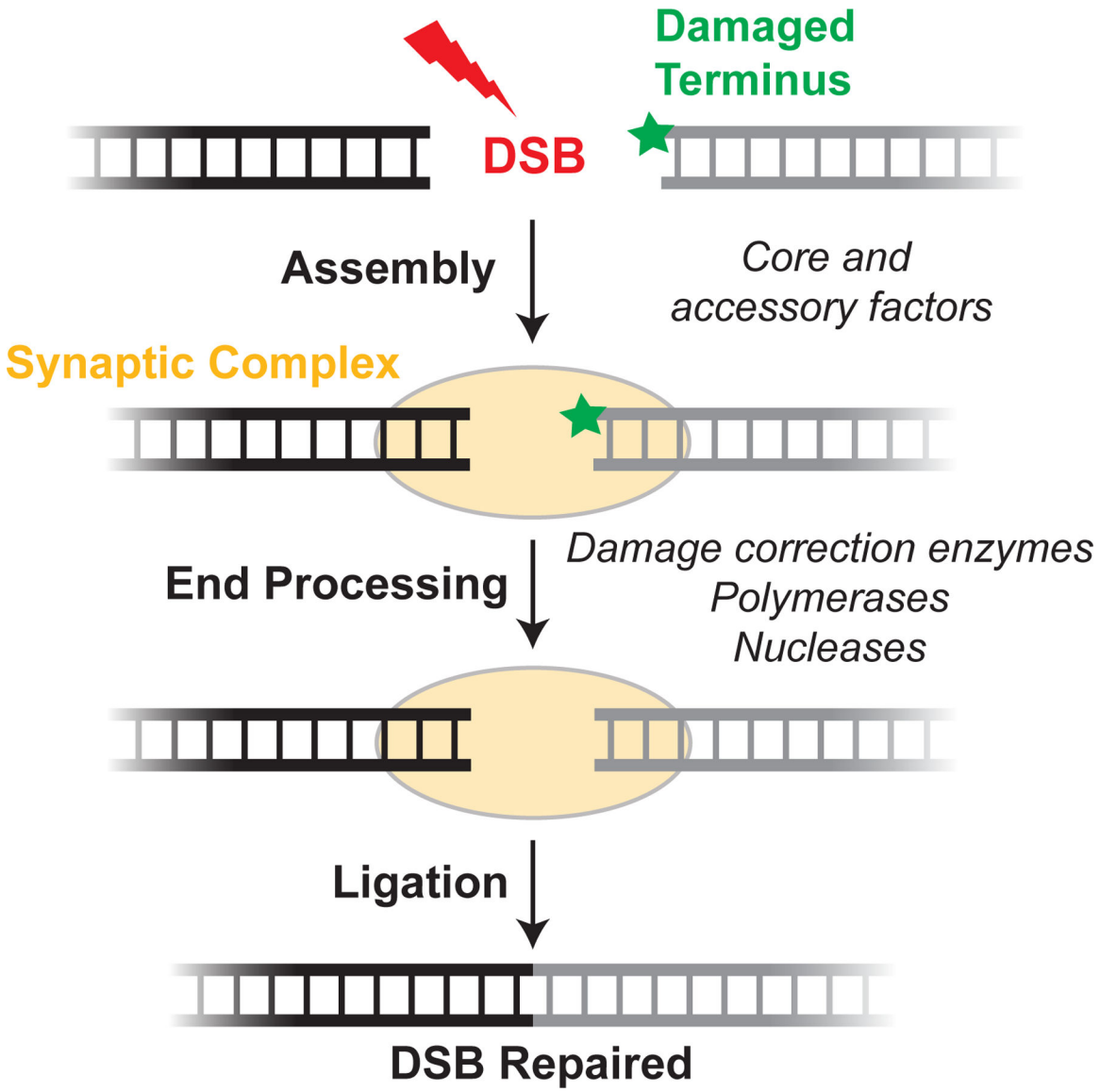

Figure 1: An overview of the NHEJ pathway

Upon DSB formation, core and accessory NHEJ factors (Table 1) recognize DNA ends and tether them together in a synaptic complex (see Section 4 for details). If the DNA ends are not compatible for immediate ligation, they are modified by processing enzymes (Table 1). until ligation can occur (see Section 5 for details).

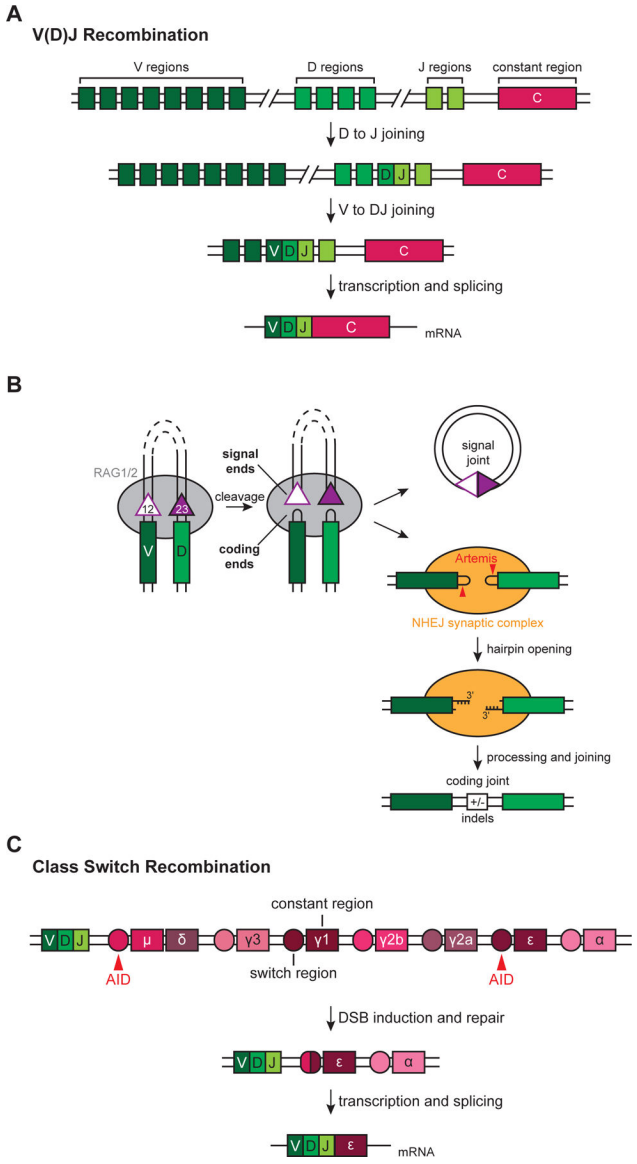

Figure 2: NHEJ and programmed DSBs

(A) Simplified example of V(D)J recombination at the IgH locus. RAG introduces DSBs adjacent to a D segment and J segment according to the 12/23 rule (see text), and NHEJ facilitates D to J joining. Subsequent V to DJ joining occurs through a similar process and assembles the V(D)J exon.

(B) Generation and repair of DSBs during V(D)J recombination. V-to-D joining is shown as an example. See text for details. 12- and 23-RSSs, open and filled purple triangles, respectively.

(C) Simplified example of CSR at the IgH locus. AID activity at the μ and ε switch regions leads to DSBs that are repaired by NHEJ in a productive class switching event to the IgE isotype.

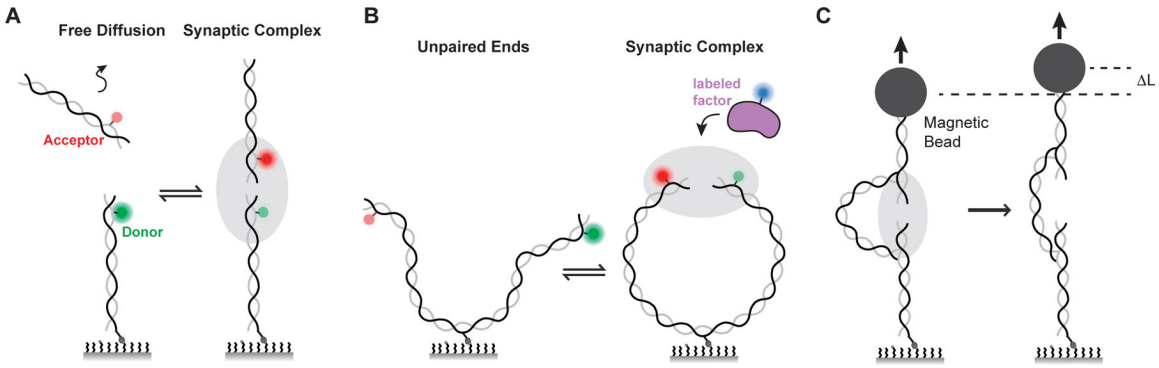

Figure 3: Single-molecule assays for synapsis

(A) Inter-molecular synapsis single-molecule FRET (smFRET) assay. Green, donor fluorophore; red, acceptor fluorophore.

(B) Intra-molecular synapsis smFRET assay. Proteins labeled with unique fluorophores (blue) can be imaged to quantify their stoichiometry and dynamics with the synaptic complex.

(C) Magnetic forceps allow application of force to a DNA scaffold during synapsis.

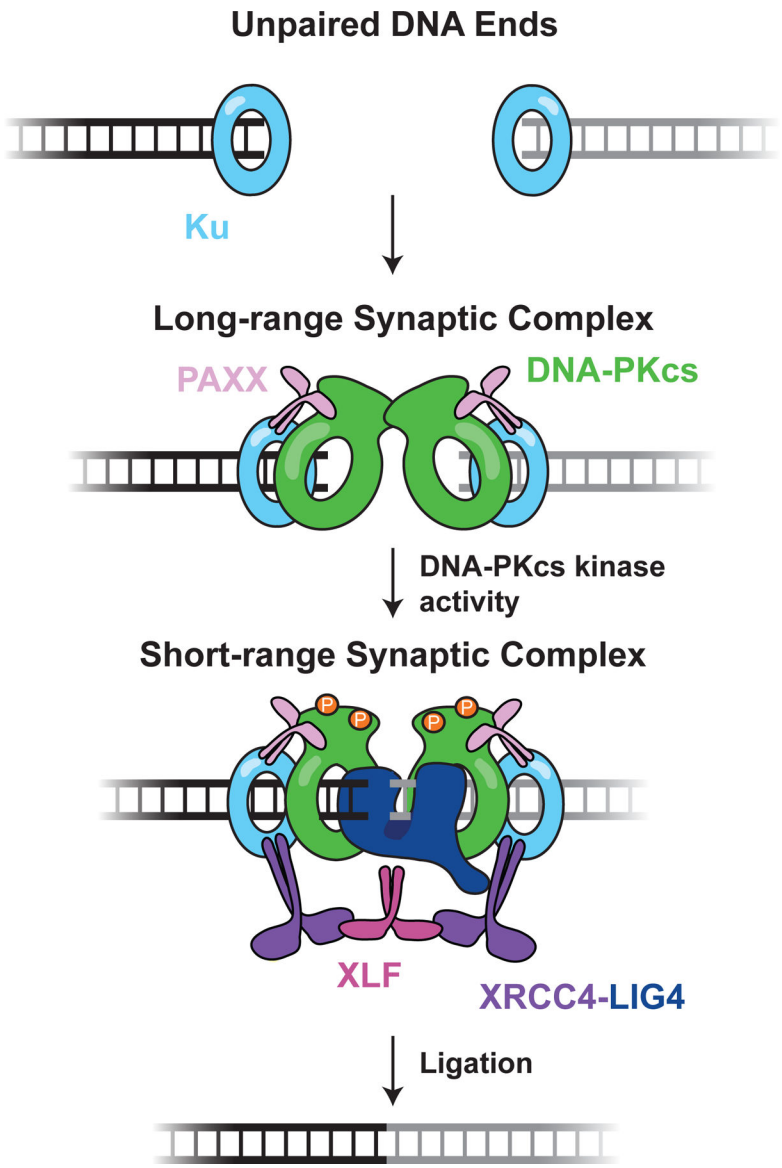

Figure 4: End synapsis during NHEJ

DNA ends are rapidly bound by Ku, which recruits further NHEJ factors. Ku, DNA-PKcs, and PAXX enable formation of the long-range synaptic complex, in which the DNA ends are tethered together but separated by >80 Å. Transition to the short-range synaptic complex, in which DNA ends are directly juxtaposed, requires XLF, XRCC4-LIG4, and DNA-PKcs kinase activity. Putative DNA-PKcs autophosphorylation in the short-range complex is shown as orange circles. Here, LIG4 is shown directly engaging both DNA ends to promote short-range synapsis, but other structural contributions are possible (see text). The figure depicts the protein and enzymatic requirements for formation of each synaptic state; the precise composition and factor stoichiometry of each state is largely unknown, beyond the observation that a single XLF dimer promotes short range synapsis (55).

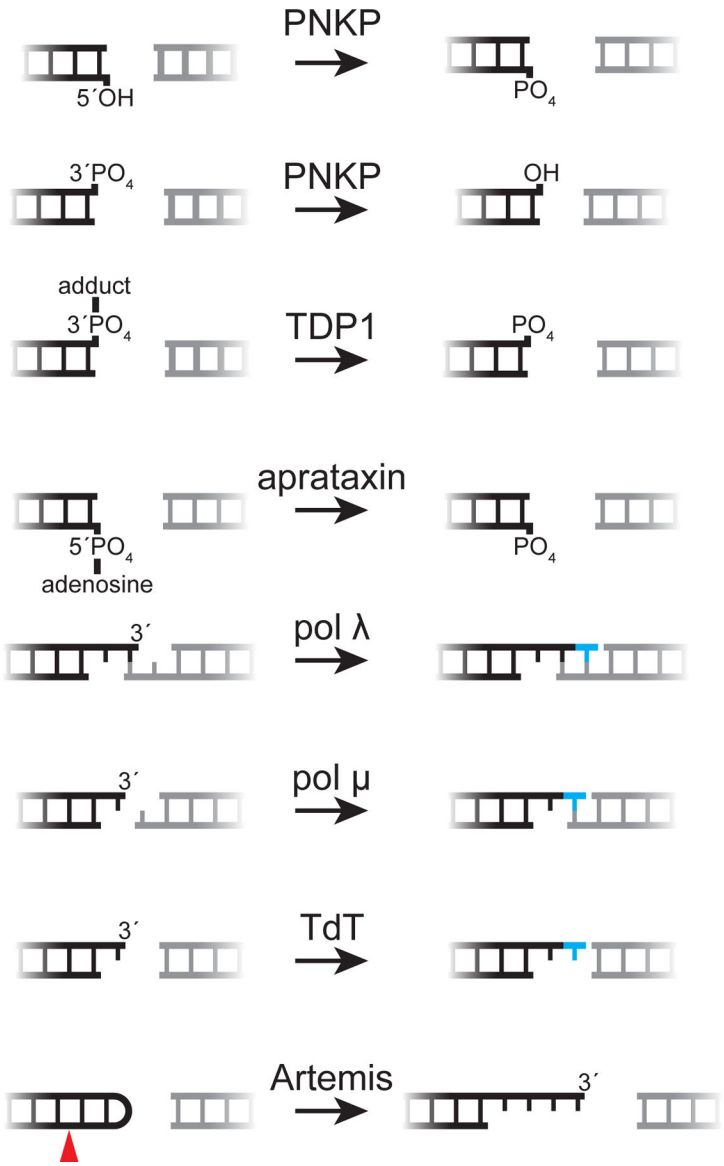

Figure 5: Processing enzymes and their DNA substrates

An array of end processing enzymes enables NHEJ to repair DSBs with diverse end structures, as shown here. Artemis is shown opening a V(D)J recombination hairpin intermediate (red arrow, cleavage site); its cognate substrates during repair of spontaneous breaks are unclear.

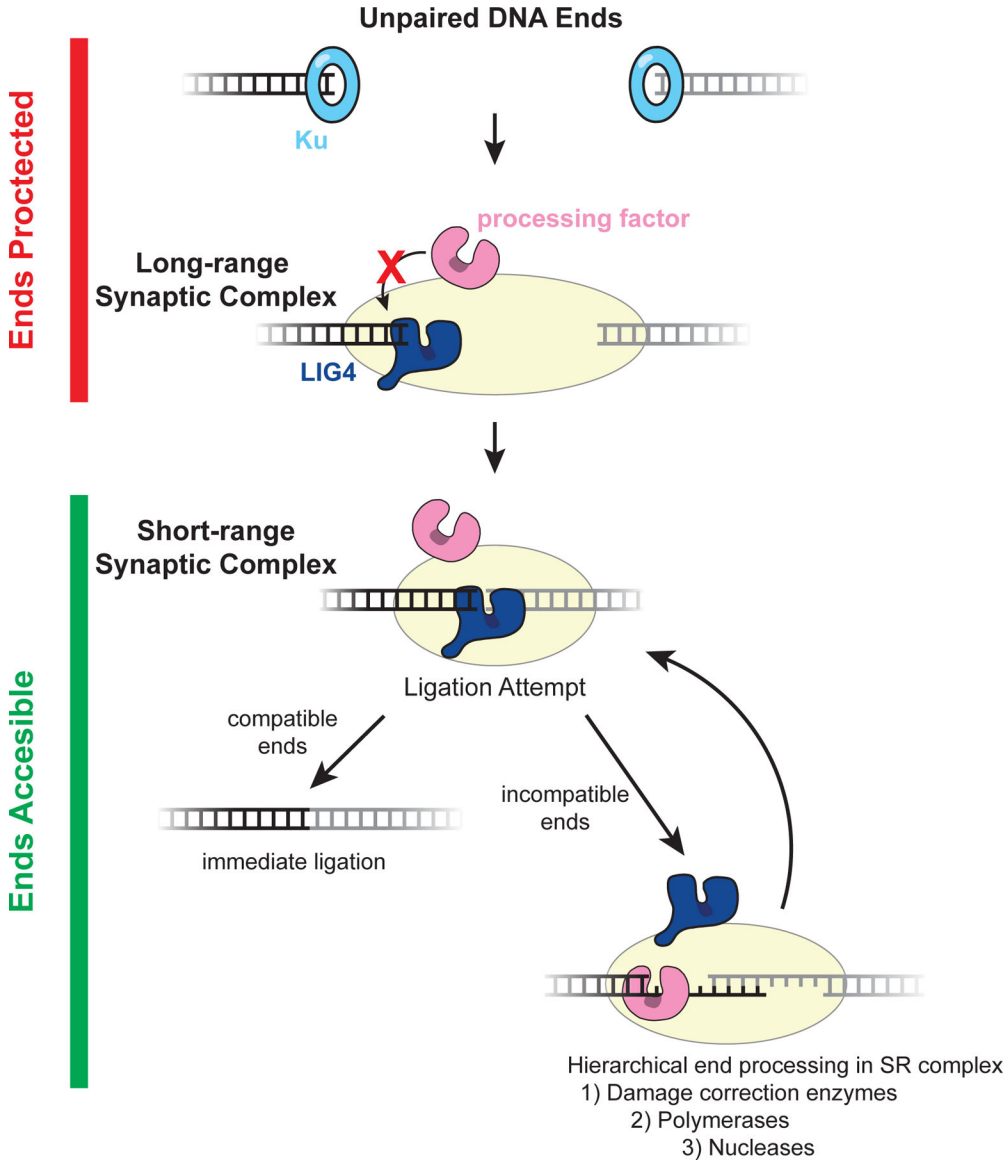

Figure 6: Model of error-prone processing regulation

Unpaired ends and those in the long-range synaptic complex are protected from aberrant or premature processing by Ku and DNA-PKcs. Although NHEJ processing factors are recruited, they are unable to act on DNA ends in these states (146). It is possible that other core NHEJ factors also protect DNA ends from processing. For example, as depicted here, LIG4 may directly engage a single DNA end prior to short-range synapsis, thereby preventing processing through steric occlusion. A second LIG4 molecule (not depicted) could protect the second end. Subsequently, direct juxtaposition of ends in the LIG4-dependent short-range synaptic enables a ligation attempt. If DNA ends are compatible, they can be ligated immediately without processing. If DNA ends are incompatible for ligation, they undergo processing in the short-range synaptic complex. Processing enzymes act hierarchically with the depicted priority to promote conservative modifications, and LIG4 frequently re-engages DNA ends to ligate them after the minimum number of processing

events required for compatibility. Together, these features allow NHEJ to repair diverse DNA ends and minimize unnecessary genomic alterations.

Table 1.

NHEJ proteins and their proposed roles.

Three classes of proteins comprise the NHEJ machinery: core factors, whose loss results in severe NHEJ defects; accessory factors, which interact with core factors but whose loss results in mild defects; and end processing enzymes, which chemically modify damaged DNA ends to prepare them for ligation.

NHEJ PROTEIN	PROPOSED ROLE(S) IN NHEJ
Core factors	
Ku70/80	Initial DSB sensor and interaction hub (11), 5′dRP/AP lyase (175)
DNA-PKcs	Kinase activity (13), end synapsis (53, 56, 69), and molecular “gate” (13)
LIG4	Catalytic role in ligation (19), structural role in synapsis (53, 56, 58, 72)
XRCC4	Constitutive LIG4 interactor (19), XLF interactor for synapsis (53, 55, 56, 58, 77)
XLF	XRCC4 interactor for synapsis (53, 55, 56, 58, 77, 80)
Accessory factors	
PAXX	Synapsis (56), redundant roles with XLF (95, 96)
APLF	Scaffolding factor (98)
WRN	Helicase and exonuclease (99), role in repair pathway choice (101)
MRI/CYREN	Cell cycle regulator of NHEJ (102, 103)
End processing enzymes	
Damage correction	
PNKP	5′ kinase, 3′ phosphatase (130)
TDP1	Removal of TOP1 (136) and other 3′ adducts (138)
TDP2	Removal of TOP2 5′ adducts (137)
Aprataxin	Removal of 5′ adenylate (132)
Polymerases	
Pol λ	Templated synthesis, paired primer terminus (127)
Pol μ	Templated synthesis, unpaired primer terminus (127)
TdT	Untemplated synthesis during V(D)J recombination (35)
Nucleases	
Artemis	V(D)J hairpin opening (32), processing of some IR DSBs (114)
SETMAR/Metnase	ssDNA endonuclease (117)