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Nonhomologous end joining: a good solution for bad ends

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Abstract

Double strand breaks pose unique problems for DNA repair, especially when broken ends possess complex structures that interfere with standard DNA transactions. Nonhomologous end joining can use multiple strategies to solve these problems. It further uses sophisticated means to ensure the strategy chosen provides the ideal balance of flexibility and accuracy.

Keywords

double strand break repair; nonhomologous end joining; DNA damage

I. Introduction

Assaults on the genome can result in various forms of DNA damage with potentially catastrophic consequences*. A particularly serious class of damage is that of the double strand break (DSB). DSBs arise during programmed recombination events such as those in V(D)J recombination, but also in response to damaging agents like ionizing radiation (IR)

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The authors declare that there are no conflicts of interest.

*Abbreviations : Alt-EJ, alternative end joining; APLF, aprataxin and polynucleotide kinase/phosphatase-like factor; ATM, ataxia-telangiectasia mutated; ATR, ATM-, Rad3-related; APTX, aprataxin; β-Casp, metallo-β-lactamase-associated CPSF Artemis SNM1 PSO2; BER, base excision repair; BRCT, BRCA1 C-terminal; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA protein kinase catalytic subunit; DSB, double-strand break; dRP/AP, deoxyribose-phosphate/apurinic/apyrimidinic; DSB, double strand break; dsDNA, double-stranded DNA; FHA, forkhead-associated; HEAT, Huntington elongation factor 2, A subunit of protein phosphatase 2A and TOR1; HR, homologous recombination; HRDC, helicase and RNase D C-terminal; IR, ionizing radiation; Ku, Ku70/80 heterodimer; MRN, Mre11/Rad50/Nbs1; NAP1, nucleosome assembly protein-1; NHEJ, non-homologous end-joining; PIKK, phosphoinositide 3-kinase-like family of protein kinases; PNKP, polynucleotide kinase/phosphatase; PAR, poly(adenyl-ribose); PARP, PAR polymerase; PBZ, PAR-binding zinc finger; ROS, reactive oxygen species; RQC, RecQ carboxy-terminal; SAP domain, SAF-A/B, Acinus and PIAS domain; SCID, severe combined immunodeficiency; SET, Su(var) Ez and Trithorax; SSBR, single-strand break repair; Tdp1, tyrosyl-DNA phosphodiesterase 1; Tdp2, tyrosyl-DNA phosphodiesterase 2; TdT, terminal deoxyribonucleotidyltransferase; vWa, von Wilebrand; WRN, Werner's Syndrome helicase; XRCC4, X ray-complementing Chinese hamster gene 4; X4–L4 complex, XRCC4–DNA ligase IV complex; XLF, XRCC4-like factor

and reactive oxygen species (ROS). DSBs that are misrepaired or left unrepaired cause genomic instability. This in turn has devastating effects at both cellular and organismal levels, including cytotoxicity, accelerated aging, cancer predisposition.

Mammalian cells rely on two major repair pathways to resolve DSBs –homologous recombination (HR) and nonhomologous end joining (NHEJ). Considered the most accurate of DSB repair pathways, HR faithfully replaces lost or damaged sequence via extensive DNA synthesis, using an intact sister chromatid as a template. The need for a sister chromatid and an abundant nucleotide pool restricts HR to the S- and G2-phase of the cell cycle [reviewed in e.g. [1, 2]]

In contrast, NHEJ directly rejoins chromosome ends, and is thus not subject to the same requirement for both homology (to use as template) and extensive synthesis, relative to HR. This makes NHEJ available for repair throughout the cell cycle [2]. On the other hand, there are disadvantages to using NHEJ. All other pathways take advantage of redundancy to replace excised damage (a sister chromatid for HR; the intact complementary strand for base excision repair, nucleotide excision repair, and mismatch repair)[3]. The inability to similarly employ sequence redundancy to replace excised damage means the standard DNA transactions – synthesis and ligation – must be made more flexible, and products of repair typically involve deletion. NHEJ thus might not be considered “DNA repair” in the strictest sense.

Compounding the difficulty for NHEJ is the mélange of possible damage at DSBs that could interfere with ligation, and, which must often be excised. DSBs generated by damaging agents are associated with a wide array of different damaged nucleotides, including oxidized nucleotides and nucleotide fragments [4–6]. Intermediates in V(D)J recombination possess hairpin-terminated breaks [7], and alignment of pairs of ends regardless of source often result in mismatches, gaps and flaps. Proteins may also occlude DSB ends, like the covalently-adducted products of aborted topoisomerase activity [8], or non-covalently associated chromatin proteins.

Thus, damage at DSBs presents a particularly onerous challenge to NHEJ. To resolve this challenge NHEJ employs a series of core factors that work to sense a DSB, align ends, and act as a scaffold for a host of processing factors that facilitate removal (and sometimes even replacement) of ligation-blocking damage (discussed in section II) (Table 1). We suggest that together this complex acts as a multi-protein machine, and is capable of employing an amalgam of the of strategies used by other repair pathways, including 1) damage tolerance, 2) targeted, short patch excision, and 3) long patch excision (discussed in section III). Having these multiple strategies at its disposal increases NHEJ’s flexibility, allowing it to resolve DSBs with diverse end structures. However, determining which strategy is used is critical, as it both impacts the fidelity and efficiency of DSB repair.

II. NHEJ is a multi-protein machine

The ability to employ several strategies for repair implies an organized, multi-protein machine capable of dynamic control of DNA end metabolism. This machine starts with the assembly of a core complex, capable of recognizing broken ends and - most importantly -

aligning ends together such that strand break termini are presented appropriately to the factors that must act on them. Notably, the core-assembly includes a pathway-specific ligase complex, which aside from its essential role it catalyzing the final step in NHEJ also provides an important non-catalytic scaffolding function.

The core assembly then recruits processing factors (Table 1). As argued above, diversity in end structure implies a need for a wide variety of end processing factors, and the list of such factors implicated in NHEJ is still growing. The list includes, not surprisingly, an array of end-cleaning enzymes shared with base excision repair/single strand break repair (BER/SSBR). Several nucleases and polymerases have also been implicated in NHEJ. However, unlike the end cleaning enzymes, nucleases and polymerases are more often uniquely suited to act in the context of a DSB and thus, are less often shared with BER/SSBR.

We discuss below the contribution of factors within the NHEJ machine as well as some gross structural features (a more detailed review of NHEJ factor structures can be found elsewhere in this issue). We emphasize how factors interact with each other and DNA substrate, as these interactions will determine how the machine is assembled as well as how the machine controls access to ends.

II.i Core factors

The core machinery for NHEJ is typically considered to include the Ku heterodimer (Ku 80/70), the DNA dependent protein kinase catalytic subunit (DNA-PKcs), DNA Ligase IV, XRCC4, and the XRCC4-like factor (XLF, or Cernunnos).

Ku—Ku is a heterodimer of 70 and 83 kD subunits (Ku70, Ku80). The subunits have a similar domain organization [9, 10] (Fig. 1A, upper panel), but N- and C-terminal domains especially have diverged in function. An extended C-terminal domain in Ku80 possesses the primary interface for promoting interaction with DNA-PKcs [9, 11, 12], while Ku70 has a C-terminal SAP domain with DNA binding activity [13]. The N-terminal domain (vWA domain) of Ku70 has acquired enzymatic activity, (see below), while Ku80's vWA domain interacts with APLF [14]. Finally, the central heterodimerization domains of both Ku subunits promote interaction with XLF [15].

Ku loads on ends by threading them through a central channel formed by the heterodimer [16] (Fig. 1A, lower panel). This binding mechanism explains its high specificity for ends and, once bound, both its ability to remain stably bound [17] and translocate internally on the DNA fragment [18].

DNA-PKcs—DNA-PKcs is a 460 kD kinase specifically recruited to, and its kinase activity dependent on, Ku-bound DNA ends [19]. It is related to two other kinases also implicated in the DNA damage response - Ataxia Telangiectasia mutated (ATM) and Ataxia Telangiectasia Related (ATR) [20]. DNA-PKcs forms a ~150 angstrom wide C clamp-like shape (Fig. 1B, lower panel) composed largely of HEAT repeats, with the kinase domain and Ku interacting region (Fig. 1B, upper panel) located as a "crown" opposite the clamp's gap [21] (Fig. 1B, lower panel). DNA-PKcs and Ku together form a very stable complex that remains tethered to the end [22, 23] and blocks access of other factors - nucleases,

polymerases, and even ligase IV - in the absence of kinase activity [23, 24]. As discussed below (section III.4) phosphorylation of DNA-PKcs, either autophosphorylation [25] or by ATM [26], plays a key role in regulating access to ends during NHEJ.

Ligase IV, XRCC4, and XLF—Ku and DNA-PKcs also recruit a pre-formed complex of ligase IV and XRCC4 [27–30]. Ligase IV includes a tri-partite N-terminal fragment (1–620) that is required for catalysis [31] (Fig. 2A). This catalytic fragment is related to the other two mammalian ligases (Ligase I and Ligase III) and likely forms a similar complex with DNA substrate, where the three domains of the catalytic fragment wrap around double stranded DNA (dsDNA) (reviewed in [32, 33]). The central portion of the catalytic fragment contains the catalytically critical lysine (K273), which is adenylated in the first step of ligation. In the second step the adenyl group is transferred to the strand break 5′ phosphate terminus, and ligation is completed when this adenylated 5′ phosphate is attacked by the juxtaposed 3′OH terminus of the strand break (reviewed in [32]).

Ligase IV is most easily identified as pathway-specific by the addition, relative to other mammalian ligases, of two tandem BRCA1 C-terminal (BRCT) domains and a short region between these two domains that interacts with XRCC4 [34–37] (Fig. 2A). XRCC4 in turn consists of a dimer with N-terminal globular heads and a coiled-coil stalk and interacts with ligase IV through a portion of the coiled-coil stalk (Fig. 2A and B, left panel) [38]. XRCC4's C-terminus, like the SSBR scaffold XRCC1, possesses a motif phosphorylated by casein kinase II that mediates interactions with related FHA domains in polynucleotide kinase/phosphatase (PNKP) [39], aprataxin [40], and aprataxin and PNKP-like factor (APLF) [41, 42] (Fig. 2A).

The XLF structure is similar to XRCC4, though the coiled-coil region is much shorter [43, 44] (Fig. 2B), and the two interact with each other through symmetrically positioned interfaces on each side of XRCC4 and XLF dimers (Fig. 2A, and C). Together with their ability to interact with DNA, they can form an extended filament (Fig. 2C) that wraps around DNA [43, 45–50] (Fig. 2D). Thus, while Ku and DNA-PKcs alone can promote the alignment of a pair of DSB ends [22], the additional recruitment of XRCC4-ligase IV and XLF and their ability to form a filament are required to further stabilize this alignment [49, 51–53].

II.ii End cleaning enzymes

End cleaning enzymes are specifically targeted to the types of ligation blocking damage that are frequently found at strand break termini. Accordingly, some of them – PNKP, aprataxin, and tyrosine phosphodiesterase 1 (Tdp1) – are employed by BER/SSBR and NHEJ both (reviewed in [54]), while others are specifically suited for DSB associated damage e.g., Ku, tyrosine phosphodiesterase 2 (Tdp2). After removing damage, these enzymes leave behind the "clean" 5′ P and 3′OH termini required for the ligation step.

Ku—Ku has 5′dRP (5′deoxyribose-phosphate)/AP (apurinic/apyrimidinic) lyase activity [55] in addition to a role in recognizing ends and acting as a scaffold for other factors. 5′dRP/AP lyase activity is required for efficient joining of substrates with near-terminal abasic sites both *in vitro* and in cells [55, 56]. Its activity is highly restricted to abasic sites

near 5′ DSB termini, and its active site is ideally located for this function [56] (Fig. 1A, upper panel). It cleaves 3′ of "standard" abasic sites (those with aldehydic isomers, i.e. products of hydrolysis or glycosylase) and generates a downstream 5′ phosphate terminus and a 3′ residue [55]. The 3′ terminal residue is typically released from ends, as Ku is active primarily near 5′ termini [55, 56]. In general, only abasic sites that would otherwise block ligation are excised and only when excision is sufficient to generate clean, ligatable termini [56]. These substrate restrictions reduce unnecessary and probably detrimental processing (e.g. by re-introducing a break after ligation).

Polynucleotide kinase/phosphatase (PNKP)—PNKP [39] like aprataxin [40] and APLF [41, 42], interacts with XRCC4 through its N-terminal forkhead-associated domains (FHA) (Fig. 3A and B).

PNKP has both 5′ kinase and 3′ phosphatase activities [57]. The kinase domain of PNKP (Fig. 3A) has a broad substrate-binding pocket that preferentially recognizes double-stranded substrates with recessed 5′ termini [58, 59]. The 3′ phosphatase domain recognizes its substrate using a deep channel, where the 3′ phosphate-containing terminus must be made single stranded and then buried [58, 60]. Of note, radiation [4, 5] and topoisomerase I can generate strand breaks with both 3′ phosphate and 5′OH termini, suggesting there are advantages to coupling both activities together on the same protein.

Aprataxin—As noted above, ligation proceeds through an intermediate where the 5′ phosphate terminus of the strand break is adenylated. Ligation can abort after adenylation, especially when termini are not readily juxtaposed, e.g., when associated with flanking DNA damage (reviewed in [32]). If the ligase releases after the aborted ligation attempt, the now adenylated 5′ phosphate is no longer a substrate for another ligation attempt. Aprataxin removes the adenylate, thus "resetting" the 5′ phosphate terminus [61, 62]. Biochemical and structural analysis indicates aprataxin is active at DSBs; and considering both its ability to physically interact with XRCC4 [40, 63] (Fig. 3A) and the probable difficulties in aligning some strand termini, it seems likely aprataxin has a significant role in NHEJ.

Aprataxin and PNKP-like factor (APLF) (also termed PALF, Xip1, and C2ORF13)—APLF has been associated with a range of nuclease activity [64, 65], including 3′ exonuclease, dsDNA endonuclease, single stranded DNA (ssDNA) 3′endonuclease activities, and AP endonuclease activity.

However, APLF's primary role may be to act as a dynamic regulator of the composition and stability of the NHEJ complex, rather than an end processing factor. As noted below (**III.iv)**, APLF may also foster substrate or factor channeling between NHEJ and BER. APLF is capable of direct protein-protein interactions with phosphorylated XRCC4 through APLF's N-terminal FHA domain [41, 42], as well as Ku through a more central region in APLF [14, 66] (Fig. 3A). These protein-protein interactions alone promote more stable retention of the ligase complex at ends, as well as ligation activity. However, APLF also promotes assembly of higher order NHEJ complexes indirectly, through its ability to bind to Poly (ADP ribose) chains (PAR) [67]. PAR is added to chromatin and repair factors near the site of strand breaks by Poly (ADP ribose) polymerases (PARPs) (reviewed in [68]), and this promotes

recruitment of APLF through a pair of tandemly repeated PAR binding zinc finger (PBZ) motifs in its C-terminus [69]. Finally, APLF has a C-terminal nucleosome assembly protein-1 (NAP1)-like motif that is sufficient for partial nucleosome assembly and disassembly in vitro [70]. This latter function may help promote local chromatin remodeling either before or after repair.

Tyrosyl DNA phosphodiesterases—DNA topoisomerases facilitate changes in DNA topology required for transcription, replication, and chromosome segregation. Changes in topology are mediated by cycles of strand-breakage and re-ligation, with the strand-break intermediate in each cycle possessing a covalent bond between a tyrosine in the topoisomerase active site and the strand break terminus (reviewed in [71]). This covalent protein-DNA intermediate can accumulate in cells when the topoisomerase cleaves within abnormal DNA structures. The causes of these structural abnormalities include damage, embedded RNA, and topoisomerase poisons (reviewed in [8]).

Topoisomerases in mammals can be distinguished by whether they cleave one strand, and form either 3′ or 5′ phosphotyrosine intermediates (Type I), or if they cleave both strands, through formation of 5′ phosphotyrosine intermediates on opposing strands (Type II). The adducted products of aborted topoisomerase activity are thus distinct; accordingly, there are two enzymes, each distinctly suited to resolve them. Tyrosine phosphodiesterase (Tdp1) releases the aborted Type I topoisomerase adduct from 3′ strand break termini [72] (Fig. 3C), while Tdp2 activity releases the aborted topoisomerase adducts from 5′ strand break termini [73].

Tdp2 can be clearly linked to NHEJ [74], consistent with the obligate association of aborted topoisomerase II activity and a DSB. By comparison, aborted topoisomerase I activity results in a single strand break (SSB). Nevertheless, Tdp1 has been implicated in helping resolve DSBs associated with type I topoisomerase adducts, e.g. as generated after replication. Tdp1 has also been linked to NHEJ through its ability to process other types of damage at DSB ends [75]. Especially relevant is the ability of Tdp1 to excise the most frequent damaged terminus found at IR-induced DSBs, the 3′ phosphoglycolate [76–78].

It is not clear how the first step in NHEJ, loading of Ku, is possible given the bulkiness of the adducted topoisomerase. Loading of Ku may thus be downstream of both ubiquitin-dependent partial proteolysis of the topoisomerase adduct [79] and, possibly, end cleaning by the tyrosine phosphodiesterase as well. Consistent with this idea, Tdp2 possesses an N-terminal ubiquitin associated (UBA) domain (Fig. 3C) that may help couple end cleaning, and eventually NHEJ, to the strand-break products of aborted topoisomerase activity [80].

II.iii Polymerases and nucleases

For the most part, each of the end cleaning enzymes implicated in strand break repair is targeted to a specific type of damage, and these enzymes target the most frequent types of damage expected given the context. However, they cannot account for all of the types of damage that can be encountered at DSBs. Moreover, end-cleaning enzymes may be unable to recognize their cognate substrate when the density or diversity of damage at an end is too great. End-cleaning enzymes are also unable to generally alter end structure, for example if

ends have gaps or mispairs remaining after end-cleaning factors have finished. Therefore, NHEJ also employs several polymerases and nucleases that can more dramatically remodel end structure.

Polymerases—There are four mammalian family X polymerases, and all can function during NHEJ when overexpressed in *S. cerevisiae* [81]. Their activities are readily distinguished in vitro: in order, pol β>Pol λ>pol μ>TdT, they possess decreasing requirement for complementary sequence opposite the primer terminus and incoming nucleotide triphosphate (Fig. 4B) [82]. The latter three can all be specifically implicated in mammalian NHEJ through specific association with Ku and XRCC4-ligase IV, as mediated by their N-terminal BRCT domains (Fig. 4A). This physical association is essential for polymerase activity at DSB ends with 3′ overhangs [82–85]. Differences in altered size and composition in "loop1", which may act as a template strand surrogate, further helps distinguish substrate specificities when comparing pol λ, pol μ, and TdT [82, 86–88] (Fig. 4C).

Pol λ—Pol λ is the most orthodox of polymerases specifically implicated in NHEJ, and is indeed also employed as a "backup" to pol β in BER/SSBR [89–91]. Nevertheless, Pol λ's ability to form a specific complex with NHEJ core factors is essential if it is to remain active in gap filling when ends are only partly complementary [82–84]. Pol λ also uniquely pinches-out (or scrunches) downstream template on gaps longer than 1 nucleotide, thereby increasing its processivity on longer gaps [92] (Fig. 4D). The latter characteristic may be particularly important in the context of NHEJ, where the end alignment needed to generate a gapped substrate is transient.

Pol μ—Pol μ activity involves synthesis using one end as primer, but in contrast to TdT it typically uses a second end as template to select a complementary incoming nucleotide triphosphate (Fig. 4B) [82, 93]. Despite this latter restriction, pol μ has no requirement for pre-existing complementarity sequence in the overhangs. Pol μ activity can thus be distinguished from both pol λ (activity requires the primer be base-paired) and TdT (activity is independent of base pairing, both opposite primer and the site of synthesis) (Figure 4B).

The roles of these polymerases in NHEJ are influenced by both differing expression patterns and partly overlapping substrate specificities. TdT is expressed only in cells active in assembling antigen receptor genes by V(D)J recombination, and its ability to diversify NHEJ repair products is consequently limited to this developmental stage [94, 95]. By comparison, Pol μ is more widely expressed than is TdT, but only at low levels [96, 97]. Like TdT, pol μ is upregulated in a cell type undergoing V(D)J recombination, but it is upregulated at a stage when TdT is not expressed - during recombination of immunoglobulin light chain (IgL) loci [98]. Accordingly, pol μ is essential for mitigating deletion during NHEJ of IgL recombination intermediates [98]. Analysis of NHEJ in pol μ-deficient mice [99] and cultured fibroblasts [100] also confirms an important role for pol μ in promoting NHEJ in non-lymphoid cell types, though it has been suggested this role is independent of pol μ's synthesis activity [100]. Pol λ appears to have a mild impact on NHEJ, especially in

non-lymphoid cell types [95], possibly because its repertoire of substrates may be a subset of Pol μ's [82].

Nucleases—A variety of nucleases have been implicated in NHEJ; notably, most are bifunctional. Artemis and Metnase are both structure specific endonucleases, while Metnase also possesses histone methyl-transferase activity. The Mre11/Rad50/Nbs1 (MRN) complex possesses structure specific endonuclease activity and exonuclease activity. Finally, the factor mutated in Werner's syndrome has both exonuclease and helicase activity.

Artemis—Artemis is the nuclease most clearly linked to NHEJ, through its role in both V(D)J recombination and radioresistance [101, 102]. Nuclease activity resides in an N-terminal domain that combines a metallo-β-lactamase fold with an appended subdomain conserved in other nucleic acid metabolizing enzymes (β-CASP domain) [101, 103–105] (Fig. 5A). A C-terminal domain contains sites for interacting with both DNA-PKcs [106–109] and the DNA binding domain of ligase IV [109, 110] and is hyperphosphorylated in cells by both DNA-PKcs and ATM after damage [106, 111, 112].

Artemis in complex with phosphorylated DNA-PKcs cleaves generally at ssDNA-dsDNA transitions, including hairpins, overhangs, bubbles, flaps and gapped substrates[113]. Cleavage in ssDNA can also be stimulated by damaged nucleotides [114].

Metnase—Metnase is uniquely found in primates, and possesses both nuclease and histone-methyl transferase activity (Fig. 5B). Both activities have been implicated in NHEJ [115, 116]. Like Artemis, Metnase cleaves within ssDNA and at ssDNA/dsDNA transitions, though there are significant differences in substrate specificity (Metnase requires a strand terminus to engage substrate) [115]. Also like Artemis, Metnase physically interacts with ligase IV [117].

Mre11/Rad50/Nbs1 (MRN) complex—MRN (Fig. 5C) performs many functions central to the DNA damage response (reviewed in [118]). Relevant to NHEJ, it can sense and bridge broken ends [119] and possesses both $3'>5'$ exonuclease as well as structure-specific endonuclease activities [120, 121]. However, as discussed in greater detail below, processing of DSB ends by MRN has been implicated in more than one DSB repair pathway.

WRN—WRN helicase and exonuclease (Fig. 5D) interacts with Ku, and this interaction both generally stimulates WRN exonuclease activity [122–125] and allows it to proceed through damaged nucleotides (e.g. 8-oxoguanine) [124]. Like MRN, WRN probably contributes to DSB end processing in multiple DSB repair pathways (discussed below).

III Strategies for resolving ends

The lack of a continuous template to guide repair factor activity distinguishes NHEJ from other repair pathways. This distinction hinders normal function of many repair factors, most obviously polymerases and ligases. NHEJ thus employs a core assembly that aligns ends together, essentially substituting for continuous template with a network of protein-protein

and protein-DNA interactions. However, this is only a partial solution, and NHEJ must flexibly employ a number of strategies for repair.

III.i Tolerance at the ligation step

The only essential step in NHEJ is the ligation of at least one strand of the DSB. Thus, one strategy presumably employed by NHEJ is the direct joining of imperfectly aligned ends via low fidelity ligation (Fig. 6A). This is conceptually similar to DNA damage tolerance during translesion DNA synthesis (TLS), which is used to bypass replication blocks caused by sites of DNA damage without the need for repair (reviewed in [126]).

There is evidence that NHEJ, at least *in vitro*, is uniquely tolerant of mismatches at the ligation step [50, 53]. Structural studies suggests this may be attributable in part to differences in how ligase IV interacts with its substrate [31], relative to other ligases. As discussed above (Fig. 2D, section II.i), though, it is probable that the incorporation of the ligase within an extended DNA-protein filament – essentially a "protein splint" to align termini together – is essential. The filament may promote ligation by simply stabilizing end synapsis, thereby increasing the time available for completing catalysis. An attractive idea is that the filament also promotes the active sampling of different alignment geometries (e.g., kinks and rotations in terminal nucleotides) and allows the ligase to take advantage of an alignment geometry that represents the lowest barrier to ligation.

However, low fidelity ligation is prone to aborting after adenylating the 5′ phosphate terminus [32]. An already adenylated ligase cannot engage this substrate; this would occur if the "spent" ligase's active site is re-adenylated *in situ*, or if the spent ligase is exchanged for another, already adenylated ligase. The former problem might be mitigated somewhat by the general resistance of ligase IV to re-adenylation, relative to other mammalian ligases [127]. Possibly more significantly, aprataxin can remove the adenylate on the substrate regardless of the reason why the ligase is also adenylated; the NHEJ complex is thus reset for another ligation attempt [61, 62].

As with translesion synthesis, a key feature of tolerance is that the junction retains damage or mismatches after NHEJ is finished (Fig. 6A). Therefore, other DNA repair pathways are required to completely restore the integrity of the DNA. Inasmuch as the retained damage may be bi-stranded, it must be carefully processing by other repair pathways (e.g. BER) to avoid re-introducing a DSB (aborted BER).

III.ii End cleaning and short patch NHEJ

DSB termini may be damaged, mispaired, or adducted such that ligation –even with reduced fidelity - is blocked. Such ends can be resolved by NHEJ using steps equivalent to those used in BER/SSBR, where repair is tailored to the specific class of damage, and excision consequently can be limited to this damage (Fig. 6B). As described above, the two pathways share some of the same enzymes (PNKP, aprataxin, Tdp1, and pol λ).

An example of "short patch repair" in NHEJ involves repair of ends with 3′ phosphoglycolates, probably the most frequent class of damage associated with IR-induced DSBs [128]. They can be resolved in cell extracts using steps borrowed from SSBR – in this

case, the nucleotide residue can be excised by Tdp1, leaving a 3′ phosphate substrate for PNKP [76–78]. The resulting one-nucleotide gap can then be filled in by polymerases μ or λ [82, 129].

The enzymes shared between NHEJ and BER/SSBR appear restricted to those involved in the late end cleaning steps expected to be common to both pathways (e.g. PNKP, Tdps). Glycosylases are required for the first step in BER (removal of damaged bases) but have yet to be linked to NHEJ and, when tested, were found to be much less active at DSB termini [130]. Base damage (e.g. oxidized bases) in some contexts can be sufficiently subtle enough to be tolerated at the ligation step (e.g. [130, 131]), thus excision may be delayed until NHEJ is complete and the junction a substrate for “true” BER.

In contrast to nucleotides with damaged bases, abasic sites cannot be tolerated at the ligation step, and the enzymes that excise abasic sites in BER (APE1, pol β) are less active at DSB ends, relative to abasic sites embedded in duplex DNA [55, 130]. NHEJ instead employs Ku [55], at least when the abasic site is near 5′ termini. Ku has a substrate specificity that is appropriately reciprocal to BER enzymes involved in abasic site removal with regard to DSB proximity [56]. Ku’s abasic site substrates can be directly generated in the same damaging event responsible for strand breakage. However, the principle source of this class of abasic site may be DSB products of an “aborted” BER reaction, where BER substrates present in opposite strands can be incised by APE1 to generate 5′dRP-terminated DSBs [132]. This mechanism is probably the primary means for generating the DSB intermediate in immunoglobulin class switch recombination [133].

As long as excision of damaged nucleotides is sufficiently limited, it may be possible to still align ends with remaining partly complementary sequence. Gap-fill-in by a polymerase could then fully reconstitute the sequence of the broken region (Fig. 6B), and this reaction has been reconstituted using cell extracts [134]. Given the parallels between this reaction and BER, the best candidate polymerase is Pol λ (Pol λ is shared with BER). As noted above, both pol μ and pol λ are active in this context (partly complementary ends that align to generate a short gap) *in vitro*, and it isn’t yet obvious if there are advantages to using one or the other.

III.iii End remodeling and Long-patch NHEJ

Targeted excision of ligation-blocking damage may not be possible, either because there is no cognate enzyme, or because the density of damage blocks the ability of an enzyme to recognize its cognate lesion. Such ends can still be resolved by resecting the damaged strand with endonucleases like Artemis and Metnase. This method of targeting damage is more flexible but less specific than short patch NHEJ (Fig. 6C cf. 6B). For example, Artemis and Metnase can excise the same 3′phosphoglycolate terminus as Tdp1/PNKP, except when nucleases are used more flanking DNA is lost and the products of excision are heterogeneous [78, 114, 135]. Since damage is removed as part of a patch (i.e. including some flanking DNA) this strategy might be considered analogous to long patch BER, or possibly nucleotide excision repair.

A long patch solution creates unique problems for NHEJ. End structures will typically be sufficiently altered or remodeled such that alignment-directed synthesis cannot restore original information, and products have deletions even if they are a substrate for direct ligation (Fig. 6B). More importantly, the odds of generating a ligation substrate after one round of nuclease activity are low. Consequently, ends often need to be subjected to cycles of repeated processing steps (iterative model) [136], whether the step involves a nuclease again or some other end remodeling factor. At the same time, the products of each processing cycle should be interrogated as a possible ligation substrate so as to avoid futile cycles and excessive sequence loss. Accordingly, Artemis interacts directly with both ligase IV and DNA-PKcs (Fig. 5A), and kinase activity of the latter is required for Artemis to be active [101, 108, 109]. These interactions suggest that DNA-PKcs may limit the activity of Artemis to an end within the context of an aligned end-pair, and physical association with ligase IV may promote a trial-ligation of the ends after each cycle of processing.

Polymerase μ probably plays an especially important role in long patch NHEJ. In pol μ's absence, excision cycles must rely on the chance generation of an overhang with sufficient complementary sequence that ligation can proceed. This can be seen as microhomology dependence, where deletion continues until identical sequence is revealed on opposite strands. Pol μ's unique ability to convert non-complementary ends to complementary ends solves this problem, as is apparent by the increased frequency of microhomology-mediated junctions observed in Pol μ's absence [82].

Like Artemis, the MRN complex also promotes a "long patch solution" to ligation-blocking damage. For example, MRN directs the release of a protein adducted to strand termini by promoting endonucleolytic cleavage of DNA a short distance (10–16 nt) away from the adducted end [137]. This is a stark contrast to the short patch solution, wherein a tyrosine phosphodiesterase cleanly removes these adducts [73].

Notably, the MRN complex may solve the problem intrinsic to long patch excision differently than suggested for Artemis. MRN at least in vitro can generates compatible ends using a "microhomology search" mechanism, rather than through cycles of processing and trial ligation. MRN can interact with a pair of ends in a synaptic complex in a manner that couples degradation of 3′ termini with the alignment of now extruded 5′ tails [138]. This presumably gives MRN the ability to sense when 5′ tails possess complementary sequence, as the exonuclease activity can be observed to pause *in vitro* as soon as complementary sequence has been revealed [139, 140]. The resulting "sticky" 5′ termini can now participate in end joining. The alternative, where each end is processed independently of the other, would be inefficient, slow, and likely require excessive deletion before a chance microhomology is revealed. In mammals, MRN performs microhomology search most efficiently with ligase III, suggesting it's role might more accurately be considered part of the alternative end joining (Alt-EJ) pathway [140].

It also seems possible that, when limited processing fails, ends are directed towards processive resection. CtIP, MRN, Exo1, and a RecQ helicase (including WRN) promote the release of Ku from ends [141] and generate long (100s of nucleotides) 3′ ssDNA tails (reviewed in [142]). These tails are typically intended to serve as the precursor to repair by

HR and can also be resolved by Alt-EJ (Fig. 6D). However, the tails could also be clipped off, perhaps by Artemis, and then resolved by canonical NHEJ. Coupling processive resection with subsequent resolution by canonical NHEJ might be aided by physical interaction between the WRN RecQ helicase and Ku [122–125], as well as the ability of DNA-PKcs to regulate WRN activity [143, 144].

III.iv Regulation of NHEJ strategy

We have proposed that NHEJ employs multiple strategies for repair, with these strategies distinguishable by both the extent and specificity of the end processing steps employed prior to ligation. How does NHEJ regulate which strategy and which processing factor within a strategy should be used?

The type(s) of damage present at the ends might rigidly determine the strategy by which NHEJ processes ends. NHEJ would thus be able to identify the specific type of damage and proceed accordingly. For example, upon recognition of an end with an adducted topoisomerase, the core assembly would specifically recruit a tyrosine phosphodiesterase. However, the complexity of damage at ends (e.g. [6]) and the number of different processing factors involved would probably rapidly overwhelm such a mechanism.

A more reasonable model is to organize processing hierarchically according to the extent of processing required. The core assembly would thus attempt to first ligate an end directly (tolerance) and proceed as needed to short patch excision, then to long patch excision (Fig. 6D). A hierarchical mechanism has the benefit of allowing for the retention of as much sequence as possible, while still eliminating the need for the NHEJ machinery to specifically identify and appropriately process all of the possible types of damage.

Transitions in processing strategies may simply occur spontaneously, as the aligned complex continues to engage a pair of ends unproductively. Processing factor recruitment and access to ends may then be somewhat stochastically determined, perhaps influenced by differing abundance of the processing factors and their affinity for substrate.

Regulation by phosphorylation—However, it is clear that changes in processing are also facilitated by dynamic reorganization of the complex as signaled by post-translational modifications. An interesting notion is that the signaling modification is not limited to the proteins involved; specifically, the 5′ adenylate product of aborted ligation or recognition of the adenylated product by aprataxin could serve as a signal to trigger a change in how the end is processed.

DNA-PKcs has already been implicated in control of processing. Phosphorylation of DNA-PKcs itself, either by itself (autophosphorylation) or by ATM, promotes first the rearrangement of DNA-PKcs at ends as well as end accessibility, then eventually DNA-PKcs dissociation (reviewed in [145]). However, there are over 30 different phosphorylation sites in DNA-PKcs [146] (Fig. 2B). Mutation of different sites has different effects on processing, indicating that there are qualitatively distinct states in accessibility (reviewed in [145]). The molecular basis for these states is not known and will require high resolution mapping of autophosphorylation sites and DNA interactions on the structure of DNA-PKcs.

DNA-PKcs also directly modifies the function of factors involved in NHEJ by phosphorylating them. DNA-PKcs (and in some instances, ATM as well) phosphorylate Artemis [106, 108, 109, 111, 112], PNKP [147], and WRN [143, 144], which in turn typically promotes recruitment and/or activity of these factors. DNA-PKcs also phosphorylates the ligase complex – most prominently XRCC4 [148] and XLF [149]. The latter phosphorylations serve to reduce DNA binding and progressively disrupt the XRCC4-XLF-ligase IV filament [150], suggesting possible intermediate states where the filament is more flexible or allows greater access for processing factors.

Regulation by ribosylation—Post-translational modification by Poly (ADP ribose) polymerases (PARPs) also affects NHEJ complex organization. PARPs are activated by strand breaks and modify factors with poly(ADP ribose) (PAR) chains [68], which directly affect the function of the factor modified and also recruit other factors that possess PAR binding modules. APLF is one such factor and, as noted above, has been shown to stabilize the assembly of NHEJ core factors at breaks directly through multiple protein-protein interactions [14]. However, APLF could also promote dynamic changes in the stability and composition of complexes present at strand breaks; its PBZ domains can interact with uncharacterized factors already present at the strand break after they are modified by PAR [67, 69]. APLF may also interact simultaneously with XRCC1 or XRCC4 [41, 42], Ku, [14, 66] and PAR [69], thus acting as a physical and inducible link between multiple strand break repair pathways (e.g. NHEJ, SSBR, Alt-EJ). This could provide a means for seamlessly re-directing repair to a different pathway. For example, APLF may promote resolution by NHEJ of aborted products of BER, or repair by BER of joined products of NHEJ that still retain damage in flanking DNA.

IV. Making the best of a bad situation

There is a wide spectrum of different end structures that can be encountered. For the fraction of these ends with simple end structures – for example, restriction enzyme-generated breaks - “simple ligation” is sufficient. NHEJ might promote fast joining of these breaks, thus preventing less savory outcomes, including the triggering of a cellular response or destruction of the initially simple end structure by a nuclease.

However, it is clear that NHEJ’s most important role is in resolving those end structures that are not already an easy substrate for ligation, and NHEJ can employ multiple strategies to still join them together (Fig. 6). We’ve suggested these strategies can be likened to those used by other pathways. The strategies include tolerance (similar to TLS), targeted excision (similar to short patch BER and SSBR), and finally a broad excision and remodeling of the initial end structure (with similarities to long patch BER, or NER). They can be ordered as above according to increasing invasiveness and consequent error, but also according to increasing flexibility, and thus capacity for more complex damage (Fig. 6D). We’ve argued therefore that NHEJ would be both most accurate and efficient when these strategies are employed hierarchically – one shouldn’t use a sledgehammer to crack a nut.

NHEJ is often considered error-prone. Such error could presumably be attributed to NHEJ's use of the wrong strategy; we suggest it is more often due to a failure to engage the broken end soon enough.

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Highlights

- We review here how diverse ends at chromosome breaks are resolved by NHEJ
- We summarize structures, interactions, and activities of factors implicated in NHEJ
- NHEJ employs an amalgam of strategies used by other repair pathways
- This gives NHEJ flexibility, but implies strategy choice must be regulated
- We speculate on why such regulation might be important

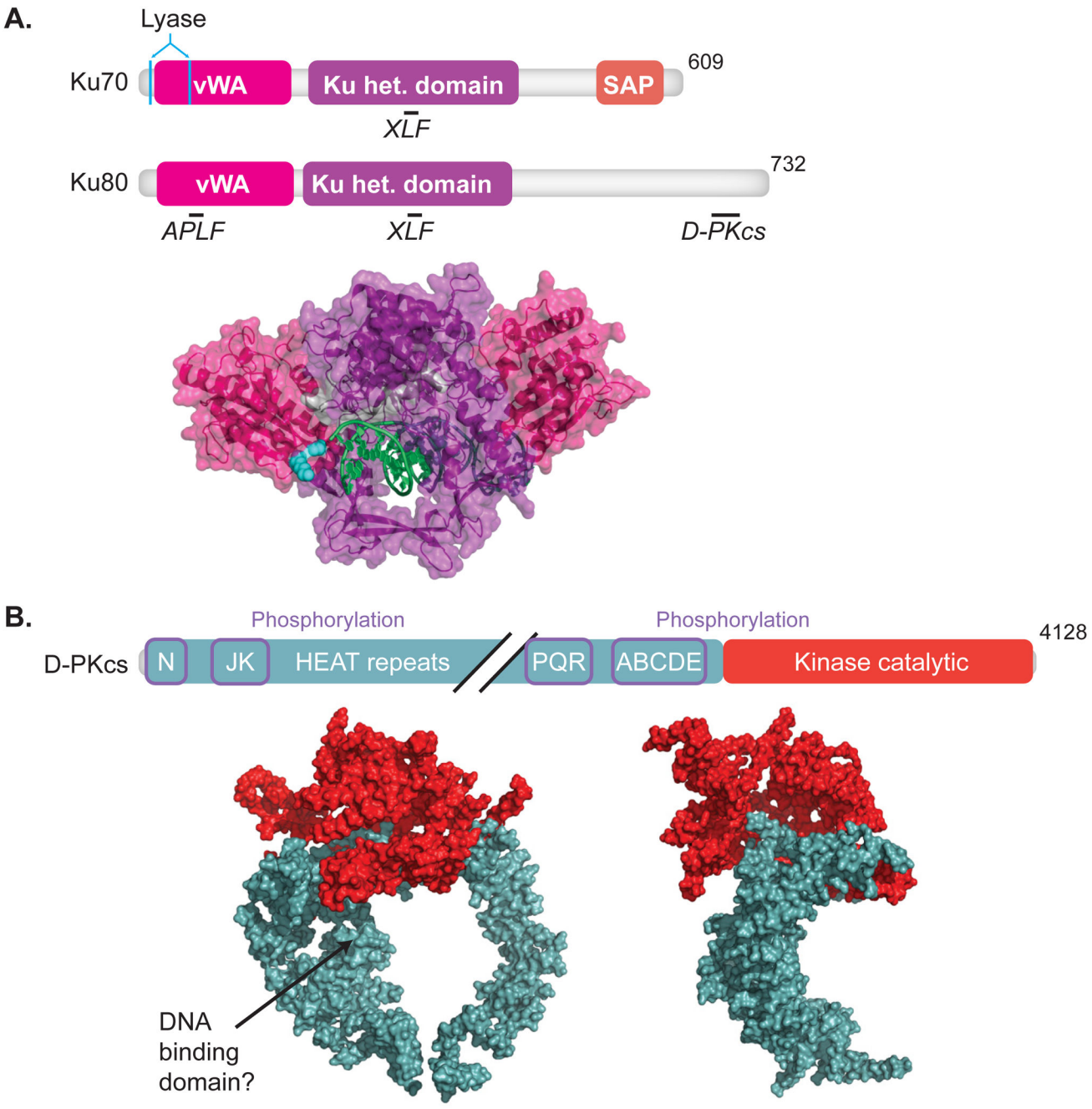

Fig.1. DNA-PK

Domain maps indicating sites of protein-protein interactions, *underlined*, interacting proteins, *italics*, (upper panel) and crystal structures (lower panel) are shown for (A) Ku70 and Ku80 (1JEY)[16] and (B) DNA-PKcs, front and side view (3KGV)[21]. Colors in crystal structures correspond to features in domain maps. (A)Domains of Ku70 include the vWA (von Wilebrand associated) domain, *pink*, the heterodimerization domain (het.), *purple*, and SAP (C-terminal SAF-A/B, Acinus and PIAS) domain, *orange*. Residues important for lyase activity are located in Ku70, *cyan* [55, 56]. Domains of Ku80 include the

vWA domain, and het. domain. Ku 70/80 interactions with APLF [14], DNA-PKcs [11], and XLF [15] are shown. (B) Domains of DNA-PKcs include an N-terminal domain, a kinase catalytic domain, *red*. Diagonal black lines denote a break in the domain map. Sites of phosphorylation, *purple boxes*, and HEAT (Huntington elongation factor 2, A subunit of protein phosphatase 2A and TOR1) repeats are indicated.

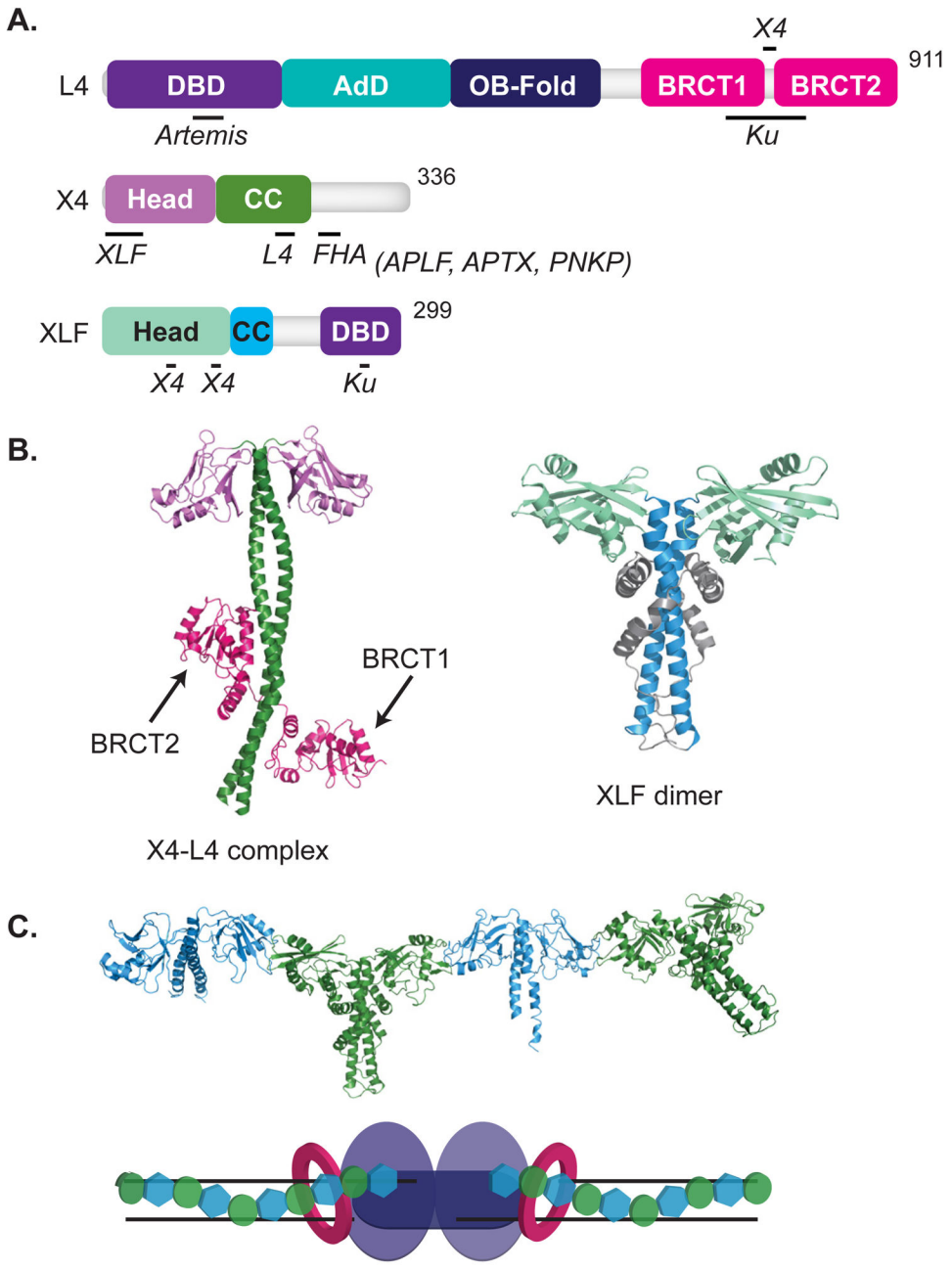

Fig. 2. Ligase Complex

Features of domain maps are as indicated in Figure 1. (A, B) Domains of DNA Ligase IV include a DNA binding domain (DBD), *purple*, adenylation domain (AdD), *teal*, oligonucleotide/oligosaccharide binding domain (OB-fold), *dark blue*, and breast cancer carboxy terminal (BRCT) domains, *pink*. Ligase IV interacts with Artemis [109], Ku [151], and XRCC4 [34]. Domains of XRCC4 include the Head domain, *lavender*, and the coiled-coiled domain (CC), *green*. XRCC4 interacts with XLF [43], ligase IV [36], APLF [41, 64], APTX [40], and PNKP [39, 152]. XLF/Cernunnos consists of a Head domain, *light green*, CC domain, *cyan*, and a DBD, *purple*. XLF associates with Ku [15] as well as XRCC4 [43].

(B) A crystal structure of XRCC4, *green and lavender*, in complex with the BRCT domains, *pink*, of Ligase IV is shown (3II6) [37]. A crystal structure of a XLF dimer is shown (2R9A) [43]. (C) Crystal structure of the filament formed by XLF, *cyan*, and XRCC4, *green*, is shown (3RWR) [45]. (D) Representation of the NHEJ core machinery (Ku, *pink rings*, DNA-PKcs, *light purple*, Ligase IV, *dark purple*) in complex with an XRCC4/XLF filament.

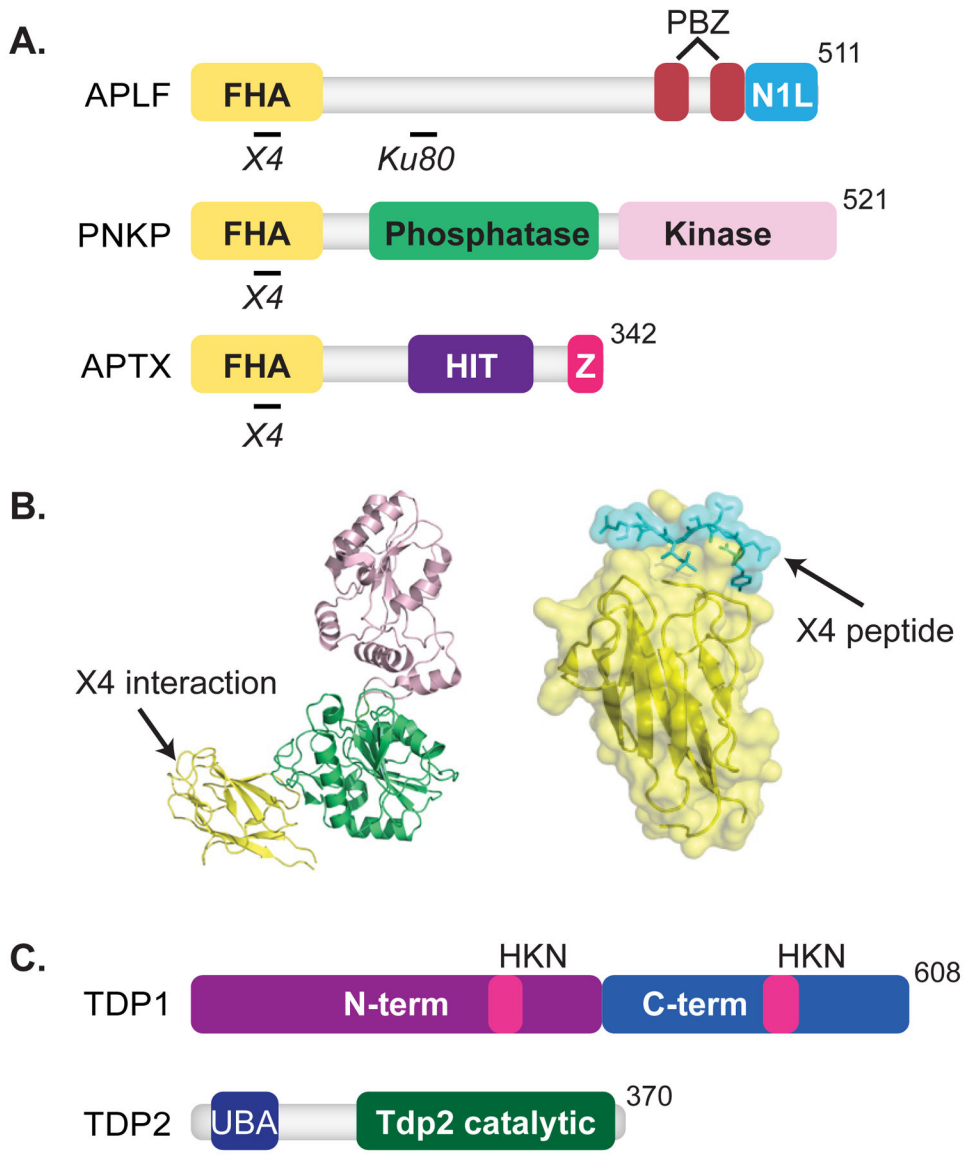

Fig. 3. End Cleaning Enzymes

Features of domain maps are as indicated in Figure 1. (A) Forkhead-associated (FHA) domains, *yellow*, are shown for PNKP, APTX, and APLF. APLF also includes two poly(ADP-ribose)-binding zinc finger (PBZ) domains, *red*, and a nucleosome assembly protein 1-like (NAP1L) motif, *blue.* APLF associates with XRCC4 [41, 64] and Ku80 [14, 66]. PNKP also contains a phosphatase domain, *green*, and kinase domain, *light pink*. PNKP interacts with XRCC4 [39, 152]. APTX contains a histidine triad (HIT) motif, *purple*, and a zinc-finger motif, *pink*. APTX interacts with XRCC4 [40]. (B) Crystal structure of PNKP (1YJ5) and the FHA domain of PNKP, *yellow*, in complex with an XRCC4 peptide, *cyan*, are shown (1YJM) [58]. (C) Domain maps of Tdp1 and Tdp2 are shown. Tdp1 consists of an N-terminal domain, *purple*, C-terminal domain, *blue*, and the catalytic HKN motifs, *pink*. Tdp2 consists of an ubiquitin-associated (UBA) domain, *blue*, and a Tdp2 catalytic domain, *green*.

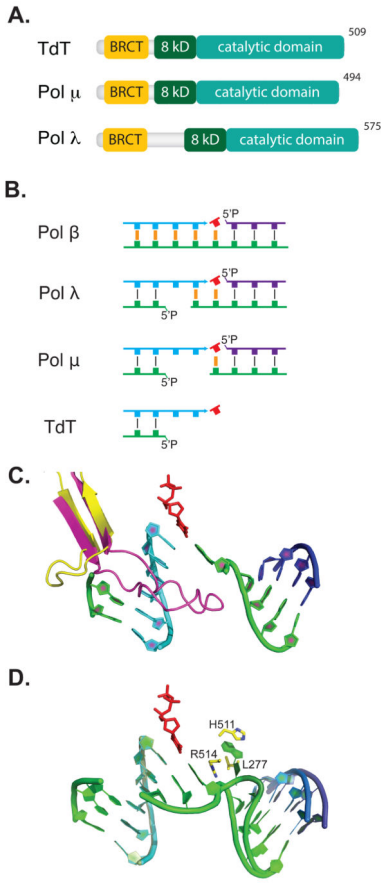

Fig. 4. Polymerases

(A) Members of X family polymerases implicated in NHEJ possess a BRCA1 C-terminal (BRCT) domain, *yellow*, an 8 kilodalton (8kD), *dark green*, and a catalytic domain, *teal*. (B) The four mammalian Family X polymerases have activity with decreasing requirement for complementary sequence, *orange*, opposite primer, *cyan*, and incoming nucleotide triphosphate, *red*. Template strand, *green*, and downstream strand, *purple*, are also shown. (C) The loop1 motif in Pol λ, *yellow*, is shown from a structure of Pol λ bound to a 1-nucleotide gap with primer, *cyan* (1XSN) [153], downstream strand, *purple*, incoming nucleotide, *red*, and template, *green*. Four nucleotides of template opposite the primer were omitted. In place of these nucleotides we show loop1 from a structure of TdT, *magenta* (1JMS) [86], aligned with 1XSN. (D) Aligned substrates from structures of Pol λ with a 1 nt (1XSN) [153] versus a 2 nt gap (3HWT) [92]; primer, *cyan*, and incoming nucleotide, *red*, are super-imposable, as is most of template, *green*, and down stream strands, *dark blue & purple*. The extra template nucleotide is buried ("scrunched") in a pocket formed by L277, H511, and R514.

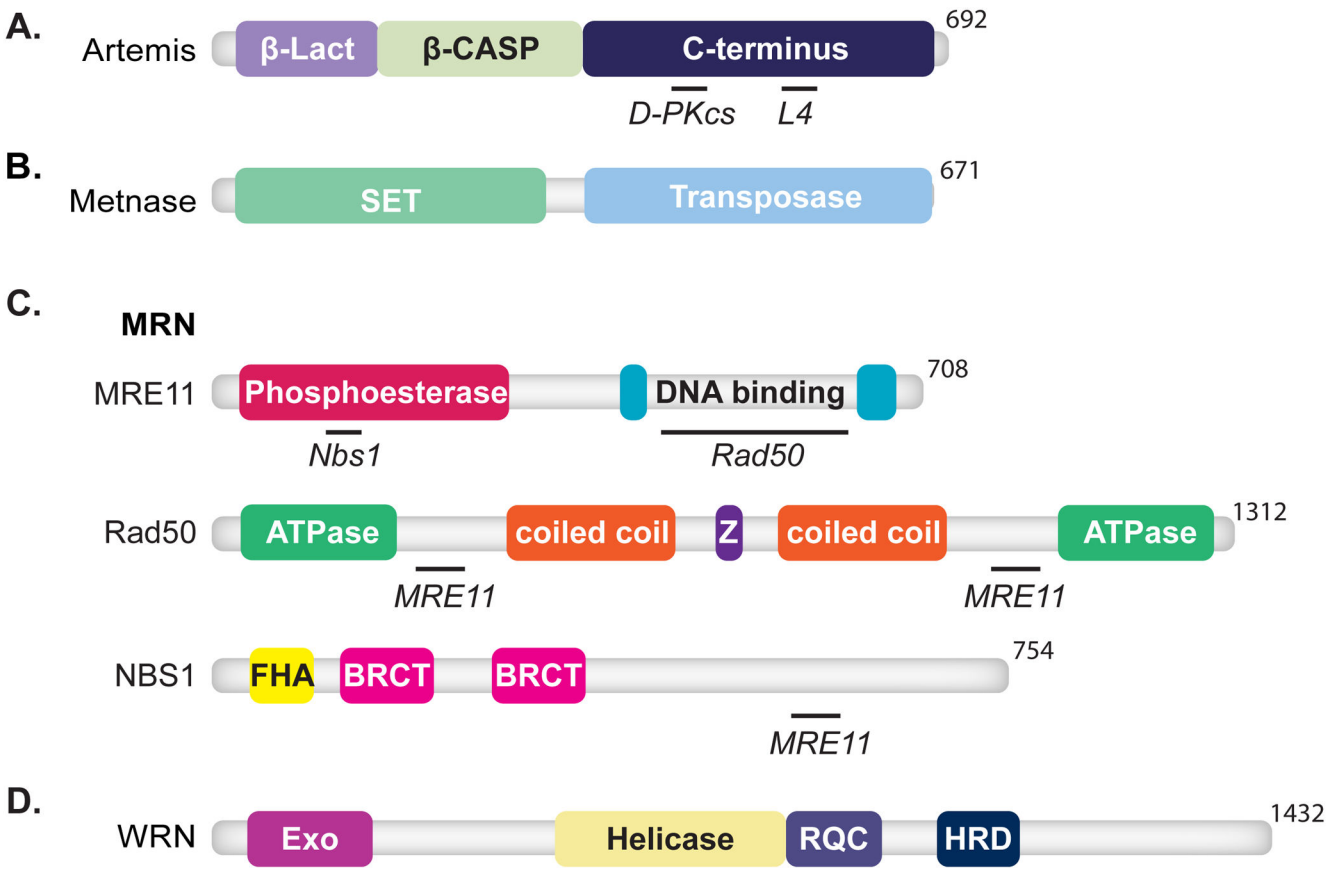

Fig. 5. Nucleases

Features of domain maps are as indicated in Figure 1. (A) Artemis consists of an N-terminal metallo-β-lactamase (β-Lact) domain, *purple*, and a metallo-β-lactamase-associated CPSF Artemis SNM1 PSO2 (β-CASP) domain, *light green*, and a C-terminal domain, *dark blue*. Artemis interacts with DNA-PKcs (D-PKcs) [106, 107] and ligase IV (L4) [109, 110]. (B) Metnase is composed of a N-terminal Su(var) Ez and Trithorax (SET) domain, *light green*, and a transposase domain, *light blue*. (C) MRN is composed of MRE11, Rad50, and NBS1. Mre11 contains phosphoesterase, *red*, and DBDs, *cyan*. Mre11 interacts with Nbs1 and Rad50 [154]. Domains of Rad50 include ATPase domains, *light green*, CC domains, *orange*, and zinc finger domain (Z), *purple*. Rad50 associates with Mre11 [154]. NBS1 consists of an FHA domain, *yellow*, and BRCT domains, *pink*. NBS1 also associates with Mre11 [154]. (D) WRN is composed of a N-terminal exonuclease domain, *fuchsia*, a helicase domain, *yellow*, the RecQ carboxy-terminal (RQC) domain, *purple*, and a helicase and RNase D C-terminal (HRDC) domain, *dark blue*.

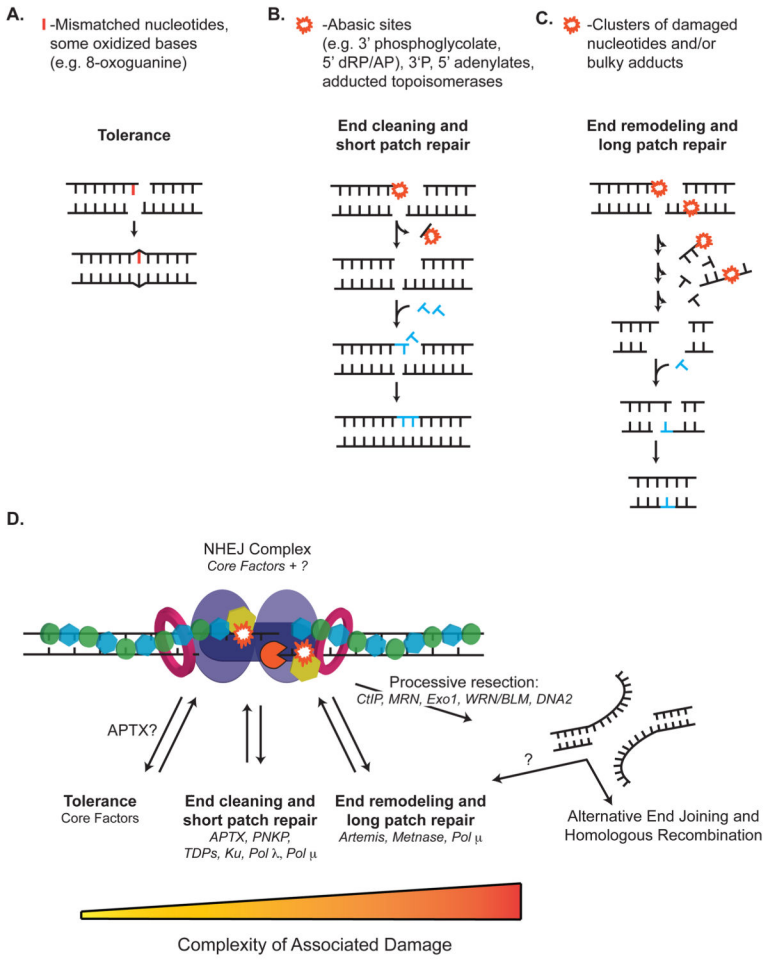

Fig. 6. Strategies for Damage Processing by NHEJ

NHEJ employs three strategies for resolving damage, *red line and starbursts*, at ends. After assembly of core factors and alignment of broken ends, the core complex can (A) attempt to tolerate the break by direct ligation, or rearrange the complex and recruit (B) end-cleaning enzymes for short patch excision, or (C) end-remodeling enzymes for more extensive damage processing. Newly synthesized and incoming nucleotides are shown, *blue*. (D) NHEJ may employ these strategies hierarchically. The choice and transition from one strategy to the next is likely determined by the complexity of damage at the ends. The NHEJ core complex is shown as described in Figure 2D.

Table 1

NHEJ associated proteins

CORE FACTORS	
Factor*	**Activity**
XRCC5, XRCC6 (Ku)	Senses DSB break [152] then recruits proteins to break
PRKDC (DNA PKcs)	Tethers broken DNA ends together [21, 22] and has kinase activity [18, 153]
LIG4 (DNA ligase IV)	Ligase [154]
XRCC4	Obligate ligase subunit [28]
NHEJ1 (XLF)	Promotes end-bridging [49]

PROCESSING FACTORS	
Factor*	**Activity**
APLF	Histone chaperone [69] 3′-5′ exonuclease, endonuclease [63, 64]
PNKP	Removes 3′ phosphates and phosphorylates 5′ hydroxyls [56]
APTX	Removes 5′-adenylate adducts [60]
TDP1	Removes Top I adducts [71], 3′ deoxyribose fragments [75, 77, 155]
TDP2	Removes Top II adducts [72]
POLM (Pol μ)	Fills in gaps when ends align with no complementarity [81]
POLL (Pol λ)	Fills in gaps when ends are partly complementary [81, 82]
DCLRE1C (Artemis)	Endonuclease, 5′-3′ exonuclease [101]
SETMAR (Metnase)	Endonuclease/exonuclease, histone methylase [116]
MRE11/RAD50/NBN (MRN)	3′-5′ exonuclease, endonuclease [120, 121]
WRN	3′-5′ exonuclease [156, 157] and 3′-5′ helicase [158]

* *HUGO* gene nomenclature: *XRCC5, XRCC6* (Ku80, Ku70), X-ray repair complementing defective repair in Chinese hamster cells 5/6; *PRKDC*, protein kinase, DNA-activated, catalytic polypeptide; *LIG4*, ligase IV, DNA, ATP-dependent; *XRCC4*, X-ray repair complementing defective repair in Chinese hamster cells 4; *NHEJ1* (XLF), nonhomologous end-joining factor 1; *APLF*, aprataxin and PNKP like factor; *PNKP*, polynucleotide kinase 3′-phosphatase; *APTX*, aprataxin; *TDP1*, tyrosyl-DNA phosphodiesterase 1; *TDP2*, tyrosyl-DNA phosphodiesterase 2; *POLM*, polymerase mu; *POLL*, polymerase lambda; *DCLRE1C* (Artemis), DNA cross-link repair 1C; *SETMAR* (Metnase), SET domain and mariner transposase fusion gene; *MRE11/RAD50/NBN*, meiotic recombination 11 homolog/RAD50 homolog/nibrin (Nbs1); *WRN*, Werner syndrome.