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The molecular basis and disease relevance of non-homologous DNA end joining

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Abstract

Non-homologous DNA end joining (NHEJ) is the predominant repair mechanism of any type of DNA double-strand break (DSB) during most of the cell cycle and is essential for the development of antigen receptors. Defects in NHEJ result in sensitivity to ionizing radiation and loss of lymphocytes. The most critical step of NHEJ is synapsis, or the juxtaposition of the two DNA ends of a DSB, because all subsequent steps rely on it. Recent findings show that, like the end processing step, synapsis can be achieved through several mechanisms. In this Review, we first discuss repair pathway choice between NHEJ and other DSB repair pathways. We then integrate recent insights into the mechanisms of NHEJ synapsis with updates on other steps of NHEJ, such as DNA end processing and ligation. Finally, we discuss NHEJ-related human diseases, including inherited disorders and neoplasia, which arise from rare failures at different NHEJ steps.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

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A DNA double-strand break (DSB) is the DNA lesion most harmful to genome integrity. Mammalian cells use two major DSB repair pathways, homologous recombination (HR) and non-homologous DNA end joining (NHEJ). Following DSB formation, the two broken double-stranded DNA (dsDNA) ends can diffuse apart. Therefore, repair proteins need to first bring the two broken ends back into proximity — a process called 'synapsis'. The synapsis step is critical, because all subsequent repair steps rely on it. The broken ends usually cannot be directly ligated, because the complexity of the end sequences first requires DNA processing (FIG. 1). In HR, strand invasion is responsible for physically aligning the damaged strand with a repair template strand; we refer the reader to other reviews for a discussion of homology-directed repair pathways[1–6].

The major pathway for the repair of DSBs in both dividing and non-dividing somatic cells is NHEJ. In NHEJ, the Ku70–Ku80 heterodimer is the first factor to bind the DSB, and it can then recruit other NHEJ proteins directly or indirectly (FIG. 1). Direct ligation of the DNA ends is performed by the XRCC4–DNA ligase 4 (LIG4) complex, an activity that is enhanced by XRCC4-like factor (XLF) and/or by paralogue of XRCC4 and XLF (PAXX). However, in many cases ligation requires DNA end processing, which can include excision, modification or addition of nucleotides. End processing relies on the kinase activity of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), nuclease activity of Artemis, nucleotide addition by polymerase-μ (Polμ) and Polλ, and nucleotide modification by tyrosyl-DNA phosphodiesterase 1 (TDP1) and polynucleotide kinase 3′-phosphatase (PNKP) (FIG. 1a). DNA end configurations, which include blunt ends, 3′ overhangs and 5′ overhangs, dictate which NHEJ proteins are required for ligation[7,8]. The differential requirement of NHEJ proteins for efficient end joining partially reflects their involvement in different end processing possibilities during NHEJ, particularly in the synapsis step[9–11]. DNA lesions at the ends also likely affect synapsis[12,13]. In contrast to HR, which usually restores the original sequence by copying from another DNA molecule, NHEJ restores the structural integrity of the DNA, but not typically its original sequence (FIG. 1).

In contrast to earlier reviews[14–16], in this Review we give particular attention to recently gained mechanistic insight into the synapsis step, which was obtained by various single-molecule methods, and place this insight in the context of the most up-to-date understanding of DNA end processing and ligation. We also discuss how DSB repair pathway choice is dictated by DNA end structures and chromatin state, and conclude with a discussion of NHEJ-related human diseases.

The choice of NHEJ for DSB repair

In human cells, NHEJ repairs almost all DSBs outside the S and G2 phases of the cell cycle. Even within the G2 phase, NHEJ also repairs as much as 80% of ionizing radiation-induced DSBs that are not close to a replication fork[17–20]. In addition to its role in repairing unscheduled DSBs, NHEJ is essential to the repair of DSBs generated during lymphocyte development[21,22], specifically in V(D)J recombination[23] (BOX 1) and during immunoglobin class switch recombination (CSR)[24]. NHEJ requires limited sequence homology (0–4 bp) between the overhangs of the broken DNA ends[15] (FIG. 2). The abundance of the Ku70–Ku80 heterodimer in primate cells (400,000 molecules per cell) and its high affinity of

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binding to the broken ends ($K_d \sim 6 \times 10^{-10}$ M) are crucial for protecting the ends from extensive resection[25].

An alternative end joining (a-EJ) pathway for repair of chromosome breaks is used mostly in cells with deficiency in key NHEJ components, such as Ku70–Ku80 or LIG4, although low levels of a-EJ can be observed in some cell types and in some organisms even when the NHEJ pathway is fully functional[26–31]. Most mammalian a-EJ requires ends that have been resected 5′→3′ (generating 3′ single-stranded DNA (ssDNA) tails longer than 20 nucleotides), followed by annealing of 2–20-bp (most often 3–8-bp) microhomologies in these tails, and action by poly(ADP-ribose) polymerase 1 (PARP1) and DNA Polθ[31–36] (FIG. 2). Other names for 'alternative end joining' (a-EJ) have been used, including 'microhomology-mediated end joining' (MMEJ) and 'alternative non-homologous DNA end joining' (alt-NHEJ), but we have not used these designations here. Given that a-EJ has its own designation, we view the designation of NHEJ as 'canonical NHEJ' as unnecessary.

Another less common DSB repair pathway, single-strand annealing (SSA), requires annealing of even longer sequences at resected ends (more than 50 bp for SSA, instead of 2–20 bp for a-EJ)[37,38] (FIG. 2). SSA requires RAD52 for the annealing step, the structure-specific endonuclease XPF–ERCC1 for removal of non-complementary tails and sealing of the remaining nick by LIG1 (REFS[37–39]). The SSA repair pathway is more error-prone than NHEJ or a-EJ because of the obligate deletion of one copy of a larger annealed repeat and the typically longer sequence between the repeats[37].

In addition to NHEJ, HR is the other major DSB repair pathway, functioning mainly in late S and G2 phases, when sister chromatids are available nearby to provide a template for repair. HR repair is usually error-free owing to the use of a donor sequence. HR repair requires extensive end resection and typically a long homology tract (more than 100 bp) as a template for initiation of repair[40] (FIG. 2).

Clearly, the extent of (micro)homology usage dictates the DSB repair pathway choice, with an increasing requirement for homology from NHEJ to a-EJ, SSA and HR (FIG. 2). NHEJ was originally named to contrast it with HR, because HR requires more than 100 bp of homology and NHEJ did not require such great lengths[41]. Although ~40% of cellular NHEJ events do not appear to require any microhomology at DNA ends, most events do involve up to 4 bp of microhomology[42]. The proportion of repaired DNA junctions that appear to not involve microhomology might actually be lower than 40% because polymerases may add nucleotides that create new microhomologies that are difficult to identify (see later).

DNA end protection is most substantial in NHEJ, because end resection is needed to expose the homology required for repair by the other DSB repair pathways. Thus, the factors that either protect the ends from resection, such as Ku70–Ku80 and TP53-binding protein 1 (53BP1), or resect the ends, such as nucleases, also contribute to DSB repair pathway choice.

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53BP1, RIF1, shieldin and other factors protect broken ends.

At the simplest DSBs — broken ends that can be directly ligated or require only limited processing, for example blunt ends or ends with compatible overhangs — end protection is possibly not crucial for favouring repair by NHEJ. For example, at a DSB that can be readily repaired by Ku70–Ku80 and rapid NHEJ activities, there may be little opportunity for complex end protection or end resection factors to gain access to the DNA ends before their joining by NHEJ. The complex interplay of protection versus resection may be relevant only to a subset of DSB lesions, including the most severe (for example, multiple breaks in close proximity), complex (for example, broken ends containing long incompatible overhangs or modified nucleotides) or long-lived DSBs, and much work is required to determine whether this viewpoint of DSB repair pathway choice is correct or not. One study suggests that the complexity of the broken ends can determine the choice of repair pathways: whereas simple DSBs are predominantly repaired by NHEJ, complex DSBs often require end resection and repair by homology-directed pathways (HR or SSA)[20].

For many breaks, DSB repair pathway choice is dictated partly by functionally opposing end resection factors, such as CtBP-interacting protein (CtIP) and the MRE11–RAD50–NBS1 (MRN) complex[43,44], and end protection proteins including 53BP1, RAP1-interacting factor 1 homologue (RIF1) and the recently identified shieldin complex[14,15,45–60] (FIG. 2). CtIP and MRN initiate end resection and generate short 3′ ssDNA overhangs. CtIP stimulates the endonuclease activity of MRN to introduce internal incisions proximal to the DNA ends. The DNA between the incision site and the end can then be degraded by MRN using its 3′→5′ exonuclease activity, thus generating 3′ overhangs. CtIP and MRN are sufficient for a short-range resection, but more extensive resection requires the nucleases exonuclease 1 (EXO1) and DNA replication ATP-dependent helicase/nuclease DNA2–Bloom syndrome protein (BLM)[1,44] (FIG. 2). Breast cancer type 1 susceptibility protein (BRCA1) and its partner CtIP can antagonize the accumulation of 53BP1 and its effector RIF1 in S and G2 phases, and can thus promote HR over NHEJ[48,61].

The DNA damage response (DDR) protein 53BP1 accumulates on chromatin around the broken ends[61]. It recognizes nucleosomes dually modified by histone H2A Lys15 ubiquitylation and histone H4 Lys20 monomethylation or dimethylation[61–63] (Supplementary FIG. 1). 53BP1 is not a factor of NHEJ repair and has no known enzymatic activities[64–66]. It protects DNA ends from resection by interacting with RIF1 (REFS[47–51]) and the shieldin complex (FIG. 2; Supplementary FIG. 1). The shieldin complex, which comprises shieldin complex subunit 1 (SHLD1), SHLD2, SHLD3 and revertibility protein 7 homologue (REV7), was recently identified to function downstream of 53BP1–RIF1 (REFS[54,55,57–60]) (Supplementary FIG. 1). It too has no known enzymatic activities. Shieldin is reported to protect the broken ends from resection and to favour the NHEJ repair pathway[57,59,60]. Cells deficient in both BRCA1 and shieldin exhibit resistance to PARP inhibitors, suggesting that deletion of shieldin switches the repair towards HR[57,59]. The regulation of REV7 dissociation from the shieldin complex by the AAA+ ATPase thyroid receptor-interacting protein 13 promotes HR, thus conferring resistance to PARP inhibitors and further suggesting a direct role for shieldin in repair pathway choice[67]. Through its role

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in protecting the DNA ends from nucleases, the shieldin complex reportedly interacts with long ssDNA (longer than 60 nucleotides) but not with dsDNA[53,55,58–60].

The molecular mechanisms of how ssDNA binding by shieldin blocks end resection are unclear. One study suggests that the conserved telomere maintenance component 1 (CTC1)–oligonucleotide/oligosaccharide-binding fold-containing protein 1 (STN1)–telomere length regulation protein TEN1 homologue (TEN1) (CST)–Polα complex functions downstream of the 53BP1–RIF1–shieldin pathway. CST–Polα-mediated DNA synthesis may protect DNA ends from extensive resection[56] (Supplementary FIG. 1). The molecular mechanisms for how the low processivity of Polα-mediated DNA synthesis could protect DNA ends are also unclear. Another study suggests that shieldin protects the ends by directly blocking end resection by EXO1, but does not specify how[68]. The shieldin complex is important for CSR but dispensable for V(D)J recombination[55,58,59,69], suggesting that its activity may be specific to certain DNA structures[58]. The overhangs formed during CSR are expected to have diverse configurations, and it is therefore unclear why the shieldin complex would be important for CSR. Moreover, the mechanism of shieldin release from the DNA overhangs to permit NHEJ end processing and repair is unclear.

The protein MRI (also known as CYREN) was first identified to interact with Ku70–Ku80 and can stimulate NHEJ ligation in vitro[70]. A recent study suggested that MRI inhibits NHEJ by interacting with the Ku70–Ku80 complex, which promotes HR during S and G2 phases[71]. Moreover, the interaction of MRI with Ku70–Ku80 occurs only in S and G2 phases, but not in G1 phase, despite MRI being normally expressed in this phase[71]. Therefore, this study suggests that MRI functions as a regulator for DSB repair pathway choice. However, another recent study indicates that MRI can interact with the Ku70–Ku80 complex during G1 phase and can stimulate NHEJ ligation in vivo[72]. MRI-deficient cells are sensitive to ionizing radiation, and also in combination with deficiency in XLF, the cells have reduced proficiency of V(D)J recombination; however, mice in which the gene encoding MRI is knocked out are normal: they do not exhibit defects in V(D)J recombination and have normal B cell and T cell development, although they have a modest defect in CSR[72,73]. These contrasting observations on the role of MRI in NHEJ are yet to be resolved.

Synapsis

Owing to the diverse types of DNA damage at DSB sites and the resulting diverse DNA end configurations, how two broken ends are brought into proximity for synapsis has been one of the most interesting aspects of NHEJ, and the most difficult to study. Application of advanced single-molecule methods has recently provided key insights into the synapsis step.

The Ku70–Ku80–XRCC4–LIG4-dependent mechanism of synapsis.

Single-molecule Förster resonance energy transfer (FIG. 3a) shows that Ku70–Ku80 and XRCC4–LIG4, which can efficiently ligate two dsDNA molecules with compatible ends in bulk solution (ensemble) biochemical studies[7,74], are also required and sufficient to mediate a flexible synapsis of two blunt DNA ends[10] (FIG. 3a,b). The flexible synapsis state discussed here is one in which the two dsDNA molecules within the synaptic complex can

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slide along each other. The use of blunt ends in experimental studies permits one to focus on the synapsis of ends that do not require alterations by nucleases or polymerases. The effect of the end sequence on synapsis is also discussed below, because transient annealing between two ends, based on nucleotide composition, affects the energetic stability of synapsis.

In the flexible synapsis state, the two dsDNA molecules are positioned side by side, aligned in parallel (FIG. 3a,b). The two DNA duplexes are dynamic because they can slide along each other in this configuration. This lateral flexibility of the DNA ends may support microhomology searching and pairing. Flexible synapsis may provide sufficient space for end processing (for example, the nucleolytic activity of Artemis) but without permitting the ends to diffuse apart. In the flexible synapsis state, the two laterally aligned ends cannot be readily ligated by XRCC4–LIG4, and other factors are needed to drive the ends into an in-line, end-to-end configuration, as described next[9–11].

XLF, which was previously reported to stimulate the ligation activity of XRCC4–LIG4 (ReFs[74–76]), can change the flexible synapsis into a structurally different synaptic state, designated the close synapsis state[10]. The two dsDNA ends within the close synapsis have in-line, end-to-end contact (FIG. 3a,b). The end-to-end-configured dsDNA molecules can be readily ligated, as confirmed by ensemble solution ligation assays[10]. XLF can interact with XRCC4 (ReFs[75,77]) and Ku80 (ReFs[78,79]), which may promote the end-to-end configuration. XLF can promote close synapsis formation either in a single step or in a stepwise manner[10] (FIG. 3b). Although PAXX can interact with Ku70 (REFS[74,80,81]), it cannot interact with XRCC4; nevertheless, PAXX can also promote the formation of a close synapsis in either a single step or a stepwise manner[10] (FIG. 3b). Compared with XLF, PAXX modestly promotes the close synapsis state. XLF and PAXX function independently to stimulate close synapsis formation, which is consistent with functional studies arguing that the role of PAXX in NHEJ is largely redundant with that of XLF[74,82]. The finding that PAXX interacts only with Ku70–Ku80 in promoting close synapsis suggests that either the interaction of Ku80 and XLF or the interaction of XRCC4 and XLF is required for the close synapsis state.

In addition to the role of NHEJ proteins in synapsis, the end sequences, which can provide transient base-stacking interactions between two ends, can also affect the synapsis. Several studies show that Ku70–Ku80 and XRCC4–LIG4 can mediate flexible synapsis for dsDNA molecules with blunt ends. Once hydrogen bonds form between the two DNA ends through chance microhomologies between the ends, Ku70–Ku80 and XRCC4–LIG4 can stimulate the formation of the close synapsis even in the absence of XLF or PAXX[9,11]. The stimulatory influence of end microhomologies indicates that any factors that can transiently stabilize the interactions at the DNA ends can promote close synapsis. This suggests that other newly identified factors, such as MRI[71,72] or intermediate filament family orphan 1 (IFFO1)[83], which can interact with Ku70–Ku80 and/or XRCC4, might be worth testing for the ability to aid the flexible synapsis to close synapsis transition.

The sequences and nucleotide modifications of the DNA ends can affect the stability of the synaptic complex. LIG4 can accommodate dynamic repositioning of ends (end remodelling)

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in a close synapsis formed by Ku70–Ku80, XRCC4–LIG4 and XLF when the DNA ends have mismatches and damaged nucleotides (for example, 8-oxoguanine)[13]. End remodelling is associated with high capacity of the close synaptic complex to directly ligate pairs of end structures that would interfere with the activity of more stringent ligases, such as LIG3 (REF.[13]).

Although some in vitro studies including those using crude cell extracts suggest that DNA-PKcs is required for NHEJ synapsis[84–87] (Supplementary Box 1), it was not found to be necessary for synapsis in other studies[9–13]. DNA-PKcs does not have a large effect on either flexible synapsis or close synapsis. The dispensable role of DNA-PKcs in synapsis is consistent with it being dispensable for in vivo formation of signal joints during V(D) J recombination[88–90] (BOX 1).

The Polμ-dependent mechanism of synapsis.

DNA Polμ belongs to the family of X polymerases. Together with the related Polλ and terminal deoxynucleotidyl transferase (TdT), it functions in DNA end processing. Polμ can mediate synapsis, as was inferred from its ability to perform template-dependent synthesis[91,92]. Efforts have been made to directly detect synapsis mediated by Polμ using a single-molecule Förster resonance energy transfer assay[11]. One study clearly showed that Polμ alone can mediate close synapsis of two 3′ overhangs sharing at least one base pair of microhomology[11]. The two dsDNA molecules are aligned in a physiological configuration within the Polμ synaptic complex (FIG. 3c). Within the synaptic complex, the two ends can be readily ligated by XRCC4–LIG4 following nucleotide addition to the ‘upstream’ primer end by Polμ. The high abundance of Ku70–Ku80 in the nucleus can inhibit Polμ-mediated synapsis if Ku70–Ku80 first occupies the DNA end. The inhibitory effect of Ku70–Ku80 is not based on interaction between Ku70–Ku80 and Polμ through the breast cancer associated carboxy terminal (BRCT) domain of Polμ. XRCC4–LIG4 can reverse the inhibition, and the Polμ BRCT domain is important for this reversion[11]. XRCC4–LIG4 may push Ku70–Ku80 inwards, away from the DNA ends, thereby exposing the overhangs and helping recruit Polμ to the ends and mediate NHEJ synapsis (FIG. 3c). The capability of Polμ to mediate synapsis is also confirmed by two recent structures of a DSB with Polμ or a chimeric polX[93,94].

Synapsis by Polμ is independent from the mechanism discussed above involving Ku70–Ku80, XRCC4–LIG4 and XLF. Relying on more than one mechanism to execute synapsis demonstrates that this step is as flexible as other NHEJ steps[14,15]. Polλ and TdT also belong to the X polymerase family, and have important functions in NHEJ[95,96]. Polλ is more abundant than Polμ in some cells and at some stages of differentiation[15]. A crystal structure of TdT bridging two DNA ends together suggests this enzyme is also sufficient to directly mediate synapsis[97,98]; the capability of Polλ to similarly mediate NHEJ synapsis is well worth testing.

Implications of synapsis flexibility for NHEJ.

Models of end processing during NHEJ have argued that it is flexible and iterative[7,14,15,99–101] (FIG. 1b); namely, that DNA ends can progress both forward and

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backward along a series of steps to modulate the repair junction before final ligation. The recent identification of two fundamentally distinct synapsis conformations — close synapsis, in which ligation can occur, and flexible synapsis, in which ends remain paired but sample alternative alignments — provides a satisfying framework within which the iterative engagement of NHEJ factors can occur[10]. Thus, even the synapsis step of NHEJ is flexible and iterative.

Whether and to what extent the engagement of these NHEJ factors is random or is determined by DNA end sequence and structure is a strong focus of current research. In one model, DNA ends are stabilized by LIG4 within the close state, but can be subtly reconfigured by LIG4 to make them suitable for ligation; the ends can partially disengage if the end structures do not favour ligation[8,13,86]. This is followed by a transition to flexible synapsis and engagement of end processing enzymes. Repair processes requiring fewer end processing iterations to generate suitable DNA ends for ligation are thus generally favoured[8]. This model helps explain why there are clearly favoured end processing steps for a given pair of end structures and sequences, especially those with existing terminal microhomologies of 1–4 bp (REF.[102]).

End processing

Natural processes that cause DSBs usually generate diverse and typically incompatible ends, which cannot be directly ligated (FIG. 1a). The ends require processing by nucleases to remove incompatible or damaged nucleotides and expose microhomology, and/or by polymerases, which add nucleotides to create a new microhomology that was not present in the original sequence. Therefore, in this respect the DNA end configurations at any specific DSB dictate which NHEJ proteins are recruited for end processing[7,8]. The nucleases and polymerases can simultaneously act on different ends of a DSB. They can also function at the same DSB during different rounds of processing (FIG. 1b). The iterative processing suggests that different sets of NHEJ factors do not exclude one another at the DNA end, and all are eligible for several rounds of junctional revision until both strands are ligated[7,14].

Nuclease activity in DNA end processing.

Artemis has intrinsic 5′ exonuclease[103] and 5′ and 3′ endonuclease activities. It is generally regarded as the main nuclease in NHEJ. In vitro biochemical studies have indicated that the Artemis–DNA-PKcs complex can markedly increase the ligation efficiency of incompatible overhangs[7,104,105]. Artemis can function on a variety of DNA end sequences, which include 5′ and 3′ DNA overhangs, blunt ends, hairpins and other substrates with ssDNA–dsDNA boundaries[106] (FIG. 4A). The trimming by Artemis of different end structures makes them suitable for ligation by the XRCC4–LIG4 complex[99,106,107] (FIG. 4Ab–Ad). At 3′ overhangs and hairpins, Artemis usually cuts and leaves a short overhang of four nucleotides at the 3′ end[106] (FIG. 4Ac). At 5′ overhangs, Artemis usually excises the phosphodiester bond at the ssDNA–dsDNA junction, leaving the ends blunt (FIG. 4Ad). On the basis of these observations, it was suggested that Artemis–DNA-PKcs binds to the ssDNA–dsDNA boundary and occupies four nucleotides along the ssDNA overhang portion of the boundary; Artemis–DNA-PKcs then cuts the DNA at the 3′ side of the occupied four nucleotides[106].

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Damaged DNA at the broken ends can also be removed by Artemis. For example, 3′-phosphoglycolates (3′-PGs), which are frequently observed at ionizing radiation-induced DSB ends[108–110], block the ligation of DNA ends. Because a hydroxy group at the 3′ end is required for the ligation step, TDP1 specifically removes 3′-PGs to allow ligation. However, cells with an inactivating *TDP1* mutation exhibit only mild radiosensitivity compared with control cells[111], which suggests that alternative enzymes besides TDP1 process the 3′-PG ends. Biochemical studies reveal that Artemis–DNA-PKcs can also remove 3′-PGs from DNA ends for efficient ligation[112,113], demonstrating that Artemis has the potential to process most ionizing radiation-induced DSBs.

The similarity in the physiological defects of Artemis-null and DNA-PKcs-null humans or engineered mice indicates that by far the major role of DNA-PKcs is activation of the nuclease activity of Artemis[90,114–117]. Mice and humans with defects in Artemis or DNA-PKcs typically lack B cells and T cells owing to failure in opening the hairpins of the coding ends during V(D) J recombination (BOX 1). These individuals are typically susceptible to ionizing radiation and DSB-inducing agents, such as bleomycin or topoisomerase II inhibitors.

Other enzymes with nuclease activity, such as aprataxin and PNK-like factor (APLF), may also have small effects on junction processing[118].

Polymerase activity in DNA end processing.

In mammals, three polymerases — Polμ, Polλ and TdT — account for most DNA synthesis activity during NHEJ[119]. All three belong to the DNA X polymerase family and interact with Ku70–Ku80 and the Ku70–Ku80–XRCC4–LIG4 complex through BRCT domains at the amino terminal of each polymerase[14,15,95,99,120,121]. Polμ and polλ are broadly expressed and act primarily to reduce the extent of deletions at NHEJ junctions by helping fill in gaps in non-complementary ends[119]. By contrast, TdT is expressed only during lymphocyte development, and introduces non-germline-encoded N nucleotides, which increase the diversity of NHEJ-assembled antigen-specific receptors[122].

The structures of DNA ends are a major determinant of polymerase activity (FIG. 4B). Strong Polλ activity requires the primer (3′) end to be double stranded (that is, the 3′ terminal base of the primer should be paired). This includes 3′ recessed ends, blunt ends and 3′ overhangs that are partly aligned and paired with 3′ overhangs of other ends[119] (FIG. 4Bb–Be). Polμ also possesses some activity on these substrates in vitro but is unable to fully compensate for Polλ deficiency, indicating Polλ activity is favoured in such DNA contexts. By comparison, whereas both Polμ and TdT retain activity on non-complementary 3′ ends (FIG. 4Bf), Polλ activity in this context is negligible[95,119].

Numerous mechanisms have been proposed to explain what determines which nucleotide is added[94,96,123,124]. Additions can occur independently of the template, with iterations of additions and nuclease activity until fortuitous addition of a complementary nucleotide leads to ligation. An alternative model suggests that nucleotides are added that are complementary to a template at another end (‘templated in *trans*’), and are thus template dependent (FIG. 4Be). There is evidence that all three polymerases can direct both template-dependent and

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template-independent additions[95,125,126], and there is consensus that the ratio of template-dependent to template-independent additions for each polymerase decreases in the order Polλ > Polμ > TdT[95]. The variation in template dependence can be attributed in part to differences in an insertion loop structure (also called 'loop 1') in the palm subdomain of the polymerases, although other structural motifs also contribute to this variation[94,96,127].

Polμ and TdT differ from conventional mammalian DNA polymerases also by readily adding ribonucleotides to DNA ends in vitro[128–133]. Moreover, in cells, both polymerases primarily add ribonucleotides during NHEJ, likely reflecting the much higher cellular pools of ribonucleoside triphosphates relative to deoxyribonucleoside triphosphates. The ribonucleotides added by Polμ and TdT are important for the ligation step, as ligation of ribonucleotide ends is more tolerant of the presence of gaps and mispairs (and likely nucleotide damage as well) in the opposite strand[133].

V(D)J recombination in human pre-B lymphoid cells can generate short (5 bp or less) inverted repeats at locally resected coding ends[42], and mouse lymphoid junctions can have direct repeats, but at a lower frequency than in humans[134]. Slippage by Polμ or Polλ may account for the formation of direct repeats[135,136] (FIG. 4Bg). Inverted repeats appear to be initiated by TdT or Polμ extension of 3′ ends; but the extended 3′ overhang may fold back, thereby generating a short inverted repeat, perhaps by Polλ[137] (FIG. 4Bh).

Four DNA ends are involved in chromosomal translocations: two ends from each of the two chromosomes involved. In lymphoid translocations, short direct or inverted repeats are sometimes derived from any of the four ends, and these sequences were originally termed 'T nucleotides'[138,139]. These repeats often have several mismatches and do not usually have microhomology at their edges. Several studies have speculated that the formation of these repeats may be due to Polμ or Polλ, similarly to the aforementioned repeats formed during V(D)J recombination[15,99,107,139].

Ligase functions in NHEJ

XRCC4–LIG4 is the sole ligase of NHEJ, and it exists in eukaryotic cells in an adenylated (energetically pre-charged) state[140–142]. Adenylated LIG4 can transfer an adenylate group to the 5′-phosphate end on one side of the strand break to generate a 5′ phosphoanhydride intermediate, which undergoes a nucleophilic attack by the 3′-OH end on the other side of the break, thus generating an intact DNA strand. In the case of a DSB, this process must occur on both strands, possibly by two independent LIG4 molecules. The adenylation of LIG4 is widely accepted to be ATP dependent. A recent claim that LIG4 can use NAD^+ for its adenylation[143] has not been reproduced by two other laboratories (B.Z. and M.R.L., unpublished results; A. E. Tomkinson, personal communication), and hence we conclude that ATP is the primary cofactor for maintaining XRCC4–LIG4 in an adenylated state.

XLF and PAXX, which exhibit structural similarity to XRCC4 (REFS[75,80,81]), can enhance the ligation activity of XRCC4–LIG4 (REFS[7,10]). XLF and PAXX have redundant roles, but XLF has higher efficiency in promoting NHEJ[74]. Although cells or mice expressing either mutant XLF or mutant PAXX exhibit a mild phenotype, mice deficient in both encoding

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genes are not viable, and *XLF*- and *PAXX*-double-knockout human lymphocytes can rarely support V(D)J recombination (*XLF* is also known as *NHEJ1*)[82,144–146]. XLF and PAXX can position two DNA ends into a structural configuration that supports ligation by XRCC4–LIG4 (REF.[10]). The structural support almost certainly involves direct interactions between XRCC4 and XLF[10]. Several studies have shown that XLF, XRCC4 and XRCC4–LIG4 can form multimeric filaments both in vitro and in cells[9,77,79,147–149]. Mutations in the Ku-binding motif of XLF that abrogate XLF–Ku80 interactions reduce filament formation and result in mildly deficient repair and radiation sensitivity[79]. Although there is evidence to suggest that the filaments may enhance pairing, synapsis and alignment of the broken ends[9], the current understanding of these filaments is rather limited. Further studies are required to define their structural, biochemical and biophysical properties, and to establish their specific functions in cells, their interaction with chromatin and the manner in which they form and localize to DSB sites.

The LIG4 complex is unusually tolerant of DNA damage and mispairs relative to prokaryotic ligases or other mammalian ligases[150–152]. Such tolerance provides important advantages to NHEJ, as cells expressing a LIG4 mutant with reduced ability to tolerate nucleotide damage are sensitive to ionizing radiation[13]. Notably, LIG4 is also more tolerant of break-flanking mispairs when the 3′ end is a ribonucleotide[133]. As discussed earlier, these ribonucleotides are frequently added by two of the polymerases active in NHEJ, Polμ and TdT.

Chromatin and condensates affect NHEJ

Biochemical in vitro studies of chromatin effects on NHEJ have been limited because of technical challenges. One study clearly showed that DNA wrapped around one histone octamer could be bound by Ku70–Ku80, even without the presence of internucleosomal linker DNA[153]. In cells, the formation of DSBs initiates complex and highly coordinated spatial and temporal processes involving chromatin, DDR factors and repair proteins. Central to these processes is the formation of a repair ‘focus’ — a designated nuclear volume in which biochemical reactions are regulated through the local chromatin environment and post-translational modifications[154–157].

The initial events in DDR signalling and NHEJ-related foci formation are still subject to much uncertainty; many of the simplest DSBs might be repaired quickly and without considerably inducing the DDR. Transient protein poly(ADP-ribosyl)ation at DSBs by PARP1 is thought to induce the formation of repair complexes[158]. The formation of these complexes is followed or accompanied by phosphorylation of the histone variant H2AX around DSB sites by the PI3K-related kinases ataxia telangiectasia mutated (ATM), ATR and DNA-PKcs[159,160], which is amplified by ubiquitylation of histone H2A Lys15 (REFS[161–163]). 53BP1 can recognize histone H2A Lys15 ubiquitylation, which facilitates the recruitment of 53BP1 and associated DDR factors, thereby changing the chromatin around DSB sites and forming unique liquid-like condensates (foci) that can affect the recruitment of repair proteins and the kinetics of repair within each focus[164]. It remains unknown how the condensate environment affects the organization of NHEJ repair complexes and regulates the repair process.

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Despite the proposed early role of PARP1 in DSB focus formation, there are conflicting reports about the contribution of PARPs to NHEJ. Several earlier studies concluded that PARP1 competes with Ku70–K80 for binding at DSBs and suppresses NHEJ[165–171], but more recent biochemical and cellular experiments have provided evidence that PARP1 activity supports NHEJ[172,173]. Additional studies are required to establish the effect of poly(ADP-ribosyl)ation — and its removal by poly(ADP-ribose) glycohydrolase — on foci formation and maturation, on the kinetics of recruitment of NHEJ proteins and on the efficiency of repair.

The recruitment of 53BP1, which is a chromatin-associated factor, to repair foci is believed to support NHEJ by suppressing HR-related DNA resection complexes through an unclear mechanism[68,154,174–177]. However, other studies have shown that in cells in S and G2 phases, 53BP1 does not suppress HR but rather promotes its fidelity[64,178]. In these cells, 53BP1 was found to suppress hyper-resection of the already resected DNA ends and prevent the error-prone SSA repair pathway, thereby promoting error-free HR[64]. 53BP1 is differentially recruited to chromatin before and after DNA replication[178], indicating that DNA replication is likely to have a decisive role in DSB repair pathway choice[19,178]. The landscapes of histone modifications and chromatin topology have recently been shown to dictate DSB repair[179–184] and 53BP1 focus mobility and fusions[185,186]. Although 53BP1 has no known enzymatic activity in NHEJ, it was proposed to have a role in promoting synapsis of DNA ends by increasing the mobility of chromatin around DSBs[187]. Further research is required to determine how chromatin features affect the recruitment of repair factors and the kinetics of repair. For more information on the effects of chromatin on NHEJ in cells, we refer the reader to recent reviews[6,188,189].

Regulation of repair by nascent long non-coding RNAs and by the cohesin complex.

An added level of complexity to the regulation of DDR and progression of DSB repair is the emerging role of RNA polymerase and its associated factors[190], which have been shown to modulate repair through the production of nascent long non-coding RNAs in the vicinity of DSBs[191–194]. These DNA damage-induced long non-coding RNAs can affect the repair process by driving the nucleation and molecular crowding of DDR proteins and repair proteins at DSBs[195,196]. The DSB-induced assembly of promoter-associated transcription machinery stimulates RNA synthesis. The synthesis of RNA could promote phase separation of 53BP1 and other DDR factors into foci, which display liquid-like condensate properties that can affect the reaction kinetics of repair proteins within foci[164,196]. Of note, several RNA-binding proteins that contain low-complexity domains have been shown to affect DSB repair and NHEJ[190,197,198]. These findings outline a complex and poorly understood relationship between NHEJ and its immediate chromatin environment.

The cohesin complex, which comprises structural maintenance of chromosomes protein 1 (SMC1), SMC3, sister chromatid cohesion protein 1 (SCC1) and SCC3 (SA1 or SA2 in humans), has important roles in DSB repair, in addition to its function in mediating sister chromatid cohesion and genome topology[199,200]. Cohesin participates in both DDR and DNA repair[201–204] — it regulates both HR and NHEJ[205–208]. In cells in S and G2 phases, cohesin was reported to prevent end joining of distant DNA ends but not of adjacent DNA

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ends, thereby preventing large chromosomal rearrangements[209]. Furthermore, the cohesin complex extrudes chromosomal DNA to form chromatin loops[210,211], which may facilitate V(D)J recombination[183,212] and CSR[184].

NHEJ-related human diseases

NHEJ is the predominant DSB repair pathway in mammalian cells. Defects in important NHEJ factors confer sensitivity to ionizing radiation. Fibroblasts from NHEJ-deficient individuals and mouse models usually exhibit marked ionizing radiation sensitivity. NHEJ-deficient humans are sensitive to both ionizing radiation and DSB-inducing chemotherapy agents such as bleomycin (Supplementary Table 1). We have compiled a list of hypomorphic NHEJ proteins identified in humans (Supplementary Table 1), which includes the mutation-related diseases and phenotypes and their molecular basis. The table also includes mouse models of NHEJ mutations and the related phenotypes (Supplementary Table 1). In this section, we briefly summarize the human mutations and their phenotypes. We also discuss the potential role of synapsis failure in contributing to neoplastic chromosomal translocations and to human diseases.

NHEJ is important for B cell and T cell development because it is required for rejoining of the broken DNA ends generated during V(D)J recombination (BOX 1). Mutation or absence of NHEJ proteins causes apoptosis of premature B cells and T cells, resulting in immunodeficiency[22,213–216]. To date, hypomorphic variants in four genes encoding NHEJ proteins — *LIG4*, *XLF*, *DCLRE1C* (encoding Artemis) and *PRKDC* (encoding DNA-PKcs) — have been identified in humans exhibiting severe combined immunodeficiency or combined immunodeficiency (FIG. 5; Supplementary Table 1). These mutations cause immunodeficiency by directly reducing protein function or by destabilizing proteins (leading to reduced protein levels, or decreasing the interactions with partner proteins). DNA-PKcs and Artemis are needed to open hairpins of V(D)J intermediates[104] (BOX 1). Cell lines from DNA-PKcs-deficient or Artemis-deficient individuals exhibit markedly reduced coding joint formation, which leads to arrest of B cell and T cell development (Supplementary Table 1). Deficiency of LIG4, which is required to ligate the coding ends (and signal ends) during V(D)J recombination causes human LIG4 syndrome with immunodeficiency[217–219] (Supplementary Table 1). XLF is not regarded as a primary NHEJ factor, although it can enhance the activity of LIG4 by promoting the formation of close synapsis[10]. Individuals with XLF deficiency exhibit immunodeficiency with impaired DSB repair and defective V(D)J recombination, and T lymphocytopenia and B lymphocytopenia are common in XLF-deficient individuals (Supplementary Table 1). However, the mouse model of XLF deficiency exhibits only a modest decrease in the number of mature lymphocytes[220] (Supplementary Table 1). This is different from the DNA-PKcs-, Artemis- and LIG4-deficient mouse models, which exhibit impaired lymphocyte development similar to that of humans with the same deficiencies.

XRCC4 interacts with LIG4 at the XID domain, thus stabilizing LIG4 and enhancing its activity[140,221]. Therefore, XRCC4 has critical roles in NHEJ repair. The XRCC4-deficient mouse model exhibits late embryonic lethality and defective lymphogenesis[222], thus showing immunodeficiency. However, no clinically significant immunodeficiency was

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observed in human patients with *XRCC4* hypomorphic variants, even in those with no detectable XRCC4 in the fibroblasts[218,223,224] (Supplementary Table 1). The basis for this specific difference in immunophenotypes in human patients versus mice is unclear.

Apart from immunodeficiencies, NHEJ deficiency can also cause other developmental abnormalities, including dwarfism and defective neurogenesis associated with microcephaly (Supplementary Table 1). Although individuals with hypomorphic variants of *XRCC4* (FIG. 5) do not have obvious immunodeficiency, they exhibit dwarfism, microcephaly, progressive neurological effects and developmental delay (Supplementary Table 1).

The relevance of synapsis plasticity to human diseases.

NHEJ is the predominant pathway that joins DSBs in configurations that cause cancer-driving chromosomal translocations in humans. It is not yet clear whether the two chromosome breaks form independently or whether a break at one chromosome mechanistically triggers a break at the other chromosome. One study described how a DSB at a single genomic location can activate the Artemis–DNA-PKcs complex[225]. While binding each of the two DNA ends at the first DSB, this activated Artemis–DNA-PKcs complex could convert a ssDNA lesion at another genomic location into a DSB, and thus create the second chromosome break.

How might an activated Artemis–DNA-PKcs complex at one genomic location encounter a ssDNA lesion at another location? We speculate this may happen owing to simple diffusion and collisions of chromosomes, although other possibilities exist. The conversion of a ssDNA lesion to a DSB by a pre-existing DSB on another chromosome would provide an explanation for the temporal and spatial coincidence of somatic cell chromosomal translocations that account for many human neoplasms. Such a mechanism may not explain all neoplastic translocations, but balanced translocations in particular might involve such a mechanism, and balanced translocations are the rule rather than the exception in haematopoietic malignancies[225].

The flexible nature of the synapsis mechanism is likely responsible for the rarity of chromosomal breaks and translocations. Deeper understanding of known and yet to be defined synapsis mechanisms and their genetic analysis in inherited and acquired diseases will be useful for understanding the rare synapsis failures that initiate translocations.

A feature of DNA end synapsis is its transient, iterative and flexible nature[9–13]. This feature is quite different from previous depictions of synapsis, which was often proposed to occur as the first step of a linear, conveyor-belt process. We have already discussed how the NHEJ mechanisms permit ends to synapse and potentially ligate, but also often to be released from one another with sufficient distance and permit further DNA end modification. This plasticity explains the variety of repair junction sequence outcomes even from initially identical DNA ends.

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Conclusion and future perspective

The nucleolytic, polymerization and ligation steps of NHEJ are all mechanistically flexible, because many different structural and chemical DNA end configurations can be ligated by NHEJ. Genetic and biochemical data suggest that NHEJ is iterative, because many repair junction sequences indicate that more than one nucleolytic, polymerization or ligation step has occurred during the joining of two ends[42,99,119,226]. Synapsis data now provide biochemical evidence for the iterative nature of this step as well.

Future insights will likely depend first on integration of biochemistry data with structural data of individual NHEJ factors, which will help elucidate their functions and explain the role of protein conformational changes in NHEJ. Second, post-translational modifications of NHEJ-related proteins would be better understood in the context of protein structure[227]. Third, the role of chromatin in NHEJ must be better understood, and this will require understanding of how histone modifications affect NHEJ. Fourth, there is much uncertainty about how DDR factors regulate NHEJ. Fifth, the molecular mechanism of how the ssDNA-binding shieldin complex prevents DNA ends from undergoing resection is far from clear. Moreover, the molecular mechanism of the release of shieldin from the DNA overhang region to allow DNA end processing and repair is also unknown. Reconstitution of shieldin-mediated end protection in vitro would provide insights into these aspects of NHEJ. Lastly, novel mechanistic insights could permit NHEJ manipulation by therapeutic agents to suppress cancer cell proliferation, and to optimize gene and genome editing or pathogen mitigation by NHEJ. Clearly, there is much work to be done.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Homologous recombination (HR).

An important DNA double-strand break repair mechanism, which usually requires long homologous sequences.

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Class switch recombination (CSR).

Recombination of the immunoglobulin heavy chain locus, which results in a switch of the expressed heavy chain isotype from IgM to IgA, IgE or IgG.

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Microhomology

Short stretches of base pairs of complementarity between two broken DNA ends.

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Synaptic complex

The complex formed by the two juxtaposed DNA ends of a double-strand break and related non-homologous end joining proteins.

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X polymerases

A subtype of DNA polymerases that includes terminal deoxynucleotidyl transferase, polymerase-μ (Polμ), Polλ and Polβ.

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N nucleotides

In V(D)J recombination, nucleotides added by the polymerase terminal deoxynucleotidyl transferase to the ends of coding DNA segments independently of a template.

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Inverted repeats

Nucleotides that are added at a DNA double-strand break repair junction and are sometimes copied inversely from either of the two broken DNA ends.

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T nucleotides

Nucleotides added at a DNA double-strand break repair junction, which are sometimes copied (directly or inversely) from either strand of either of the two broken DNA ends in a template-dependent manner.

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Box 1 |

Roles of non-homologous DNA end joining in V(D)J recombination

During B lymphocyte and T lymphocyte development, V(D)J recombination rearranges the variable regions of antigen receptor genes, which include variable (V), joining (J) and possibly diversity (D) segments[21]. V(D)J recombination includes two distinct phases: the V(D)J recombination activating gene 1 (RAG1)–RAG2-mediated cleavage phase and the non-homologous DNA end joining (NHEJ)-mediated joining phase[21,107]. In addition to the RAG1–RAG2 endonuclease complex, high mobility group protein B1 (HMGB1) is also thought to participate in the cleavage phase. RAG1–RAG2 recognizes the recombination signal sequences (RSSs), which consist of 12RSS and 23RSS (see the figure), and synapses them (this is in contrast to the sequence-independent synapsis of two broken DNA ends during repair by NHEJ). the RSS consists of a heptamer and a nonamer sequence, and a linker sequence between them. whereas the heptamer and nonamer sequences are conserved, the linker sequence is usually random and consists of either 12 or 23 bp (hence 12RSS and 23RSS). the recombination process follows the ‘12/23 rule’, in which one 12RSS and one 23RSS are typically required. Once the 12/23 synapsis is formed, RAG1–RAG2 first nicks one strand of each RSS at the boundary between the coding flank and the heptamer sequence of the V, J and in some cases D segments, then it mediates the formation of the hairpins at the coding ends and leaves the signal ends blunt[231,232].

Following the RAG cleavage phase, Ku70–Ku80 can bind to any of the four DNA ends (coding and signal ends) and recruit the Artemis–DNA-dependent protein kinase catalytic subunit (DNA-PKcs) complex. The Artemis–DNA-PKcs complex binds to the coding ends, initiates a series of protein phosphorylations and activates Artemis. One recent study suggests that hairpin DNA ends cause autophosphorylation of DNa-PKcs at its ABCDE sites, but not at its PQR sites[227]. The phosphorylation of the ABCDE sites would then allow DNA-PKcs to activate Artemis, presumably through a conformational change in DNA-PKcs that triggers a conformational change in Artemis. Activated Artemis, which is an endonuclease, opens the hairpins[104]. the now open-ended DNA can activate DNA-PKcs to autophosphorylate its PQR sites. This fully phosphorylated DNA-PKcs can now also phosphorylate the carboxy terminus of Artemis. the opening of the hairpins by Artemis–DNA-PKcs usually leaves a 3′ overhang at the coding ends[106]. Once the hairpins are opened, the coding ends can be subjected to common NHEJ end processing, which includes further end resection by Artemis and nucleotide additions by DNA polymerases (polymerase-μ (Polμ), Polλ and terminal deoxynucleotidyl transferase (TdT)). TdT is T cell and B cell specific, and adds most of the junctional nucleotides in a template-independent manner, which is a major factor leading to junction diversity; local nucleotide resection (less than 15 bp from each end) is another major factor of junction diversity[42]. The ligase complex includes XRCC4-like factor (XLF), XRCC4 and DNA ligase 4 (LIG4)[107]. RAG1–RAG2-dependent NHEJ repair is different from the common RAG1–RAG2-independent NHEJ, because the coding ends generated during V(D)J recombination are hairpins and require opening by the Artemis–DNA-PKcs complex

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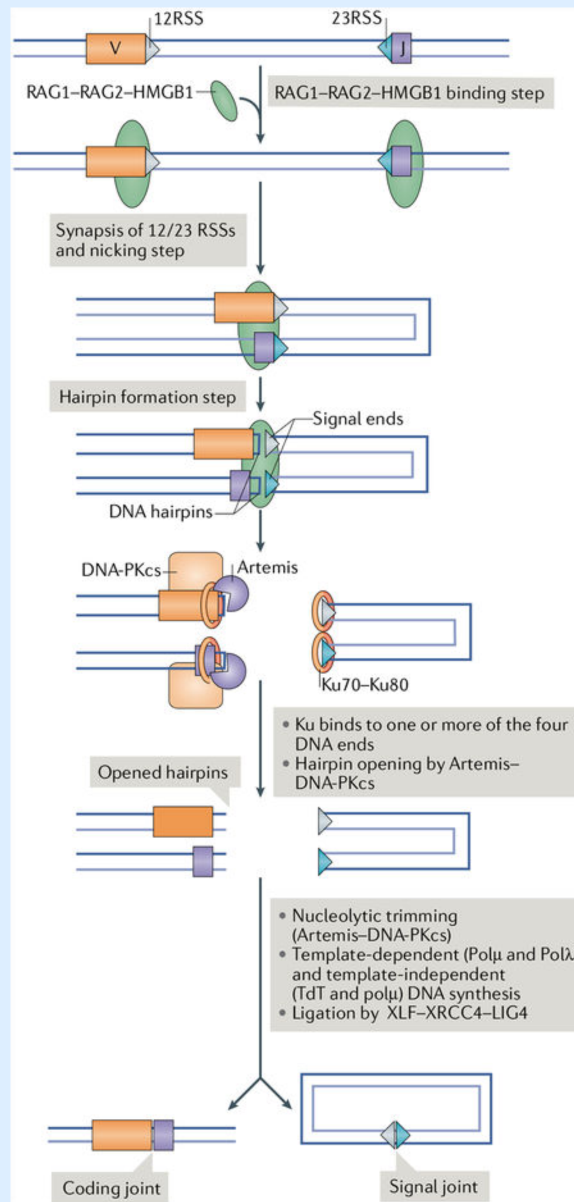
before NHEJ repair. Moreover, the RAG1–RAG2 complex can hold the four ends before transferring them to NHEJ repair[233].



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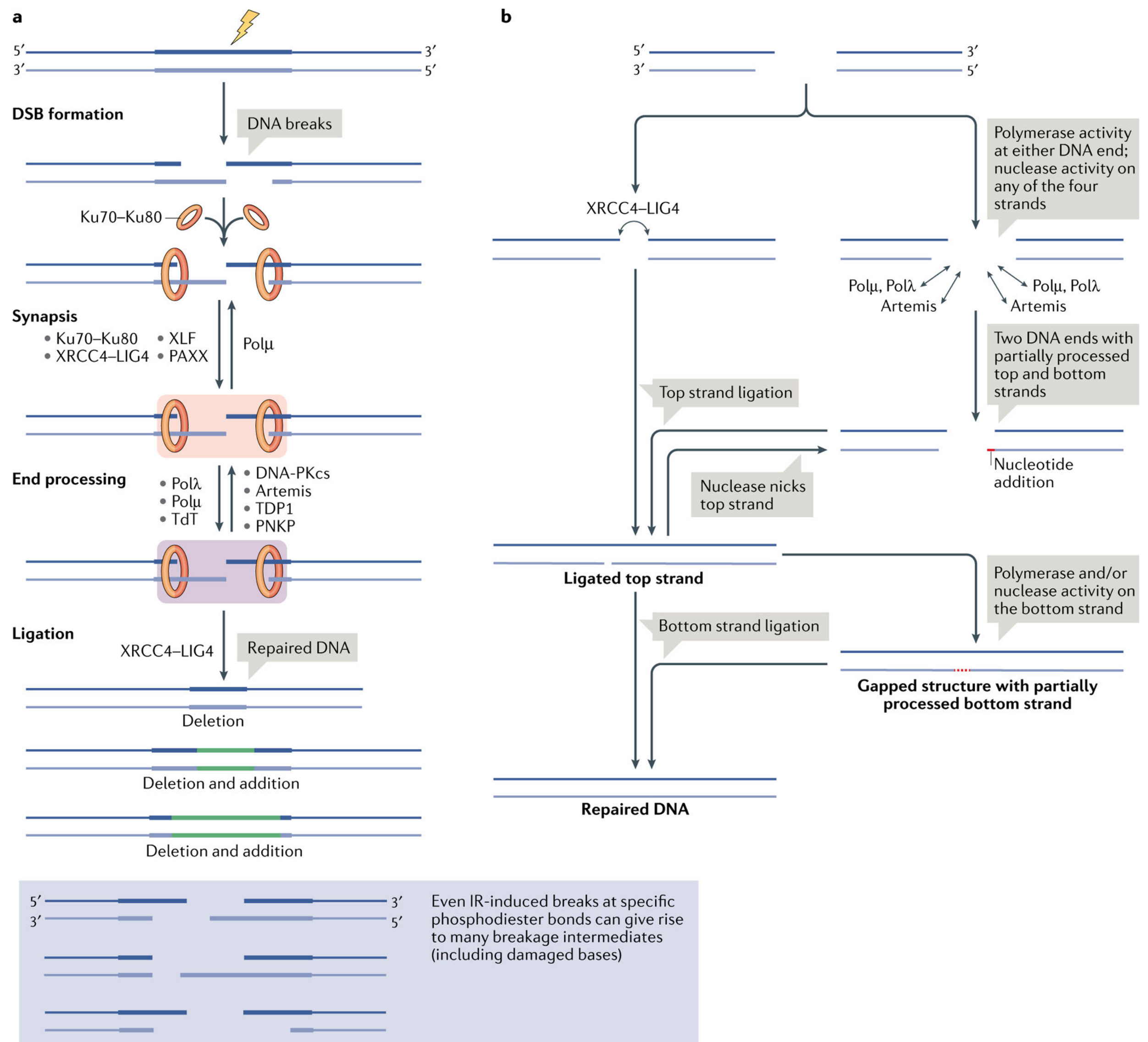

Fig. 1 |. Overview of the non-homologous DNA end joining process.

a | Non-homologous DNA end joining (NHEJ) begins with binding of the Ku70–Ku80 heterodimer to the ends of the double-strand break (DSB). The biochemical steps of NHEJ include synapsis, which brings the diffused DNA ends back into proximity, end processing and finally ligation. Two independent mechanisms exist for NHEJ synapsis. One depends on Ku70–Ku80, XRCC4–DNA ligase4 (LIG4), XRCC4-like factor (XLF) and/or paralogue of XRCC4 and XLF (PAXX). The other depends on DNA polymerase-μ (Polμ). Synapsis (pink box) is depicted in detail in FIG. 3. DNA ends that are incompatible for direct ligation by LIG4 are processed by the nuclease Artemis or by polymerases (Polμ, Polλ and terminal deoxynucleotidyl transferase (TdT)) to become compatible for ligation. Artemis and tyrosyl-DNA phosphodiesterase 1 (TDP1) can remove 3′-phosphoglycolates (not shown), which block ligation and can be generated at DSBs caused by ionizing radiation (IR). End processing (lilac box) is presented in detail in FIG. 4. Naturally occurring DSBs almost always feature sequence alterations at the DNA ends, even before their modification by

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NHEJ factors (blue box). Together, the diverse nature of the damage ends and of end processing give rise to diverse repair junctions, including small deletions and insertions, although precisely joined products are also found at low frequency, especially when the ends are compatible for direct ligation[228,229]. The green lines represent added nucleotides. **b** | The end joining process is flexible and iterative, meaning that DNA ends with diverse configurations can be covalently ligated following various modifications. XRCC4–LIG4 can ligate each strand independently of the other. The Artemis–DNA-dependent protein kinase catalytic subunit (DNA-PKcs) complex can trim overhangs to expose complementary regions and can also nick a gap at the ligated strand. The nicking of the ligated strand would generate the same or modified DNA ends, possibly with overhangs for another round of end joining. DNA polymerases (Polμ and Polλ) can add nucleotides to either create microhomologies or to fill in gaps to facilitate DNA strand ligation. Nucleotide addition by polymerases may also generate a flap (not shown), which requires endonucleolytic cleavage by Artemis. The iterative nature of NHEJ allows multiple rounds of revision. PNKP, polynucleotide kinase 3′-phosphatase.

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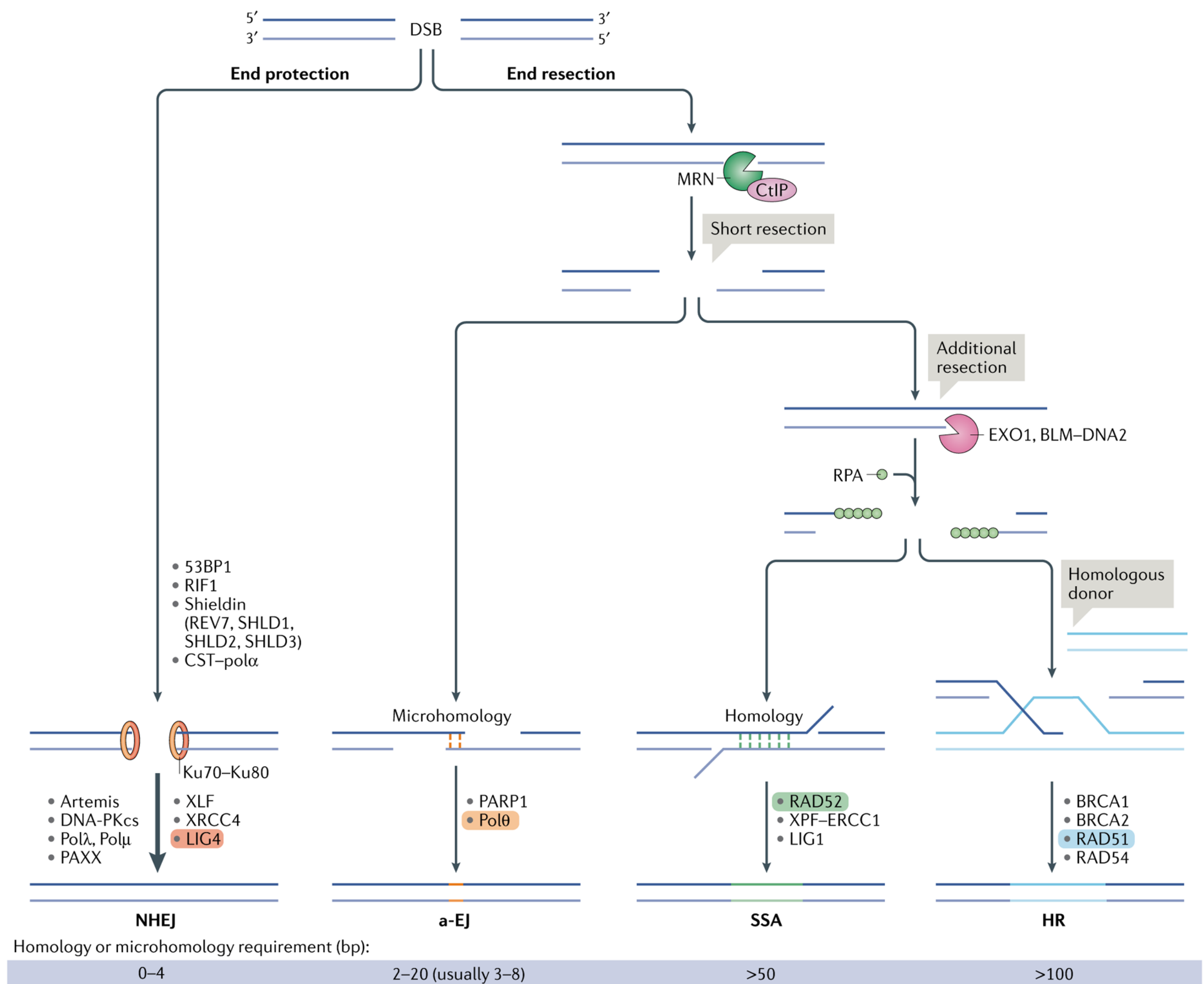

Fig. 2 |. DSB repair pathway choice.

DNA double-strand breaks (DSBs) are repaired by non-homologous DNA end joining (NHEJ), alternative end joining (a-EJ), single-strand annealing (SSA) and homologous recombination (HR). NHEJ is the predominant DSB repair pathway (bold arrow). Pathway choice is largely dictated by the availability of homology (called microhomology if the length is less than 20 bp) between the DNA end overhangs. NHEJ requires either no microhomology or, more often, 1–4 bp of terminal microhomology. a-EJ typically requires microhomology of at least 2 bp (usually more) and less than 20 bp. SSA requires homology of typically more than 50 bp, and the homology requirement for HR is typically more than 100 bp. The exposure of terminal (micro)homology is partly determined by the extent of DNA end protection versus nucleolytic resection. TP53-binding protein 1 (53BP1) and its effectors, RAP1-interacting factor 1 (RIF1), the shieldin complex (comprising shieldin complex subunit 1 (SHLD1), SHLD2, SHLD3 and revertibility protein 7 homologue (REV7)), the conserved telomere maintenance component 1 (CTC1)–oligonucleotide/ oligosaccharide-binding fold-containing protein 1 (STN1)–telomere length regulation protein TEN1 homologue (TEN1) (CST) complex and polymerase-α (Polα) protect the ends from extensive resection. By contrast, CtBP-interacting protein (CtIP) and the MRE11–

RAD50–NBS1 (MRN) endonuclease first nick one strand near the 5′ end and then degrade the strand in a 3′ to 5′ direction towards the end, thereby creating a short 3′ overhang, which is suitable for a-EJ. Poly(ADP-ribose) polymerase 1 (PARP1) and Polθ are important for a-EJ. The nucleases Bloom syndrome protein (BLM)–DNA replication ATP-dependent helicase/nuclease DNA2 and exonuclease 1 (EXO1) can mediate longer resection in a 5′ to 3′ manner, and replication protein A (RPA) protects the resulting single-stranded DNA (ssDNA) to facilitate HR and SSA. Annealing of homologous sequences by RAD52 is important for SSA, and the 3′ non-homologous ssDNA flaps are cut by XPF–ERCC1 before ligation by DNA ligase 1 (LIG1). RAD51, breast cancer type 1 susceptibility protein (BRCA1), BRCA2 and RAD54 are essential to promote HR. The (micro)homology regions within the repair products are labelled with colour; the proteins highlighted with colour are those essential for the corresponding pathways. DNA-PKcs, DNA-dependent protein kinase catalytic subunit; PAXX, paralogue of XRCC4 and XLF; XLF, XRCC4-like factor.

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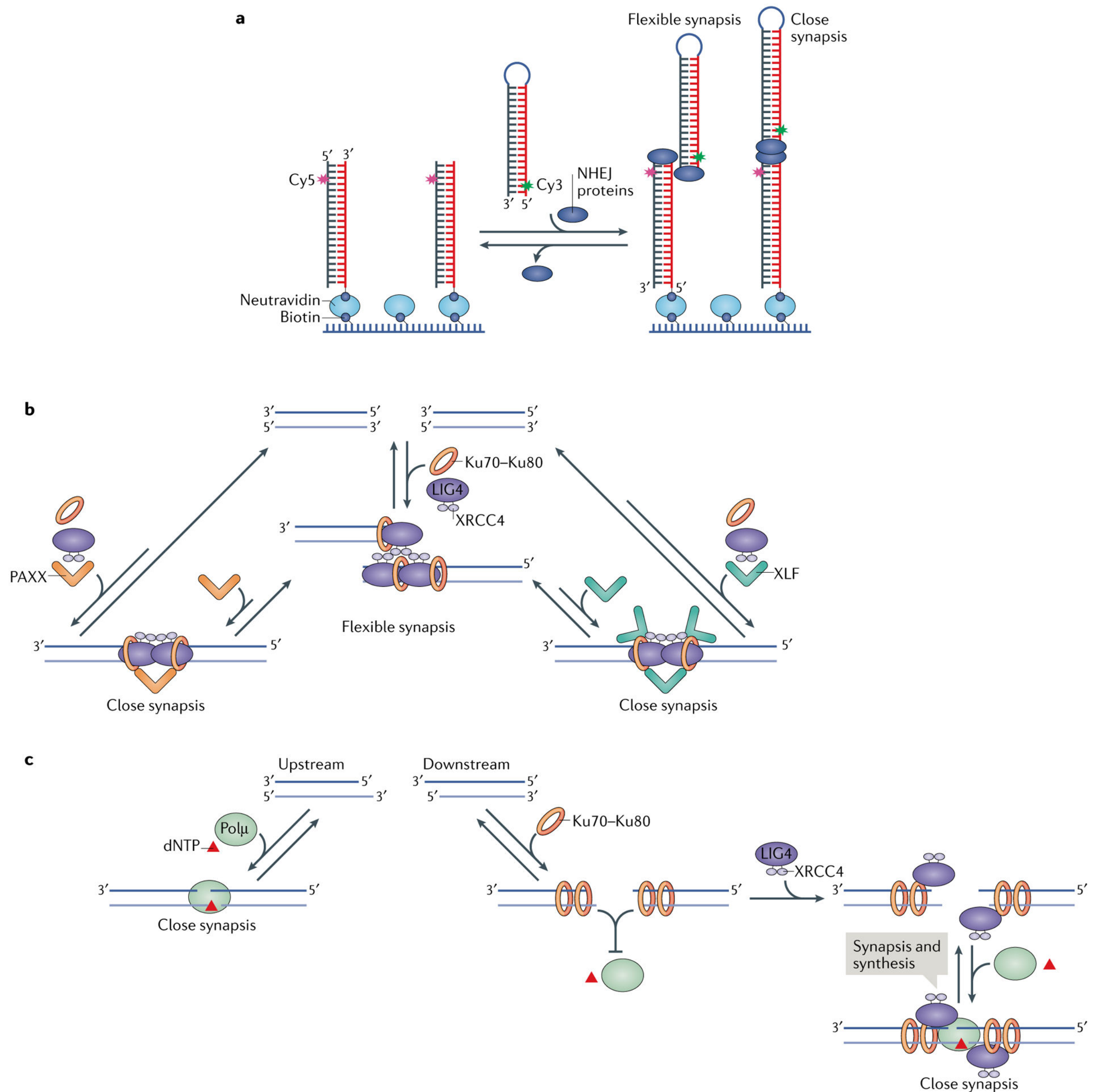

Fig. 3 |. Mechanisms of NHEJ synapsis.

At least two mechanisms exist for non-homologous DNA end joining (NHEJ) synapsis: a Ku70–Ku80–XRCC4–DNA ligase 4 (LIG4)–XRCC4-like factor (XLF)-dependent mechanism and a DNA polymerase-μ (Polμ)-dependent mechanism. The choice of synapsis mechanism depends on the configurations of the DNA ends and the availability of different NHEJ proteins. **a** | Single-molecule Förster resonance energy transfer for synapsis analysis. A fluorescently labelled DNA molecule is immobilized on a slide, and a differently labelled DNA molecule together with NHEJ proteins is then added onto the slide to initiate synapsis. **b** | The Ku70–Ku80–XRCC4–LIG4–XLF-dependent mechanism of synapsis. Two structurally different synaptic complexes corresponding to flexible synapsis and close synapsis are formed through this mechanism. In flexible synapsis, the two duplexes are

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laterally aligned; flexible synapsis can be mediated by Ku70–Ku80 and XRCC4–LIG4 for both blunt ends and overhangs. XLF and/or paralogue of XRCC4 and XLF (PAXX) can promote the close synapsis in either a stepwise manner, in which they drive the two duplexes from the lateral configuration (flexible synapsis) to an end-to-end close contact configuration, or in a single step, in which the close synapsis is immediately formed by Ku70–Ku80, XRCC4–LIG4 and XLF or PAXX. When short terminal microhomologies exist between the overhangs, Ku70–Ku80 and XRCC4–LIG4 can also promote close synapsis in the absence of XLF and PAXX (not shown). The two duplexes within the close synapsis can be readily ligated by XRCC4–LIG4. **c** | The Polμ-dependent mechanism of synapsis. Close synapsis of DNA ends with 3′ overhangs and short microhomology can be mediated by Polμ. Nucleotide addition can then occur within the close synapsis. High abundance of Ku70–Ku80 can inhibit Polμ-mediated synapsis if Ku70–Ku80 occupies the DNA ends first. XRCC4–LIG4 can reverse this inhibition, possibly by pushing Ku70–Ku80 inwards along the DNA, thereby exposing overhangs and helping recruit Polμ to mediate NHEJ. Not shown are the 5′ overhang configuration, because it can be either easily trimmed by Artemis or filled in by Polμ or Polλ to generate a blunt end; filament formation — for chromatinized DNA, filaments might be important for synapsis[9]; and a suggested role for DNA-dependent protein kinase catalytic subunit in synapsis[84–87] (Supplementary Box 1). dNTP, deoxyribonucleoside triphosphate. Parts **a** and **b** adapted from REF.[10], Springer Nature.

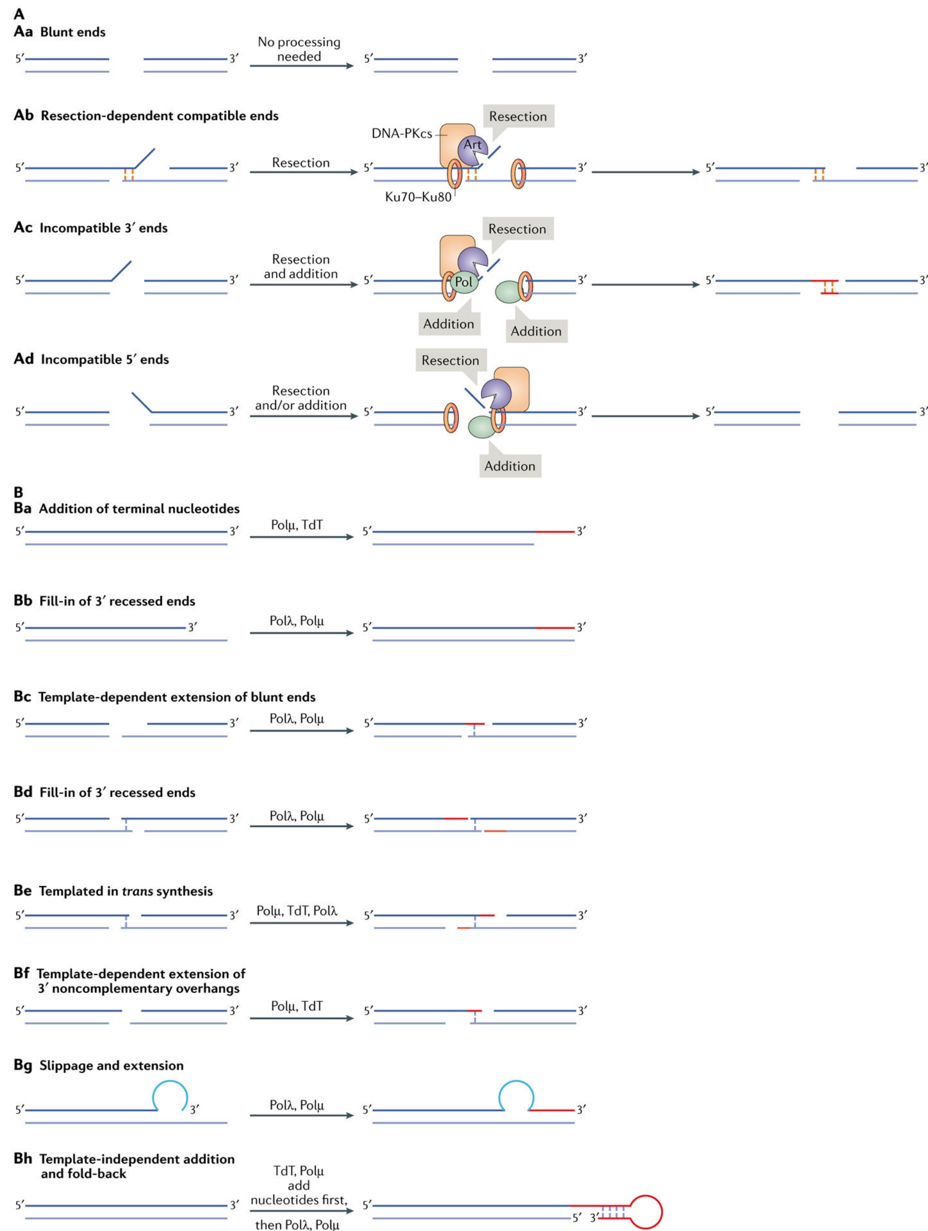

Fig. 4 |. Various NHEJ end processing mechanisms.

a | Resection of broken DNA ends with different configurations. **aa** | Blunt ends are often readily ligated and repaired without processing. **ab** | At resection-dependent compatible ends, the nuclease Artemis, which interacts with and is activated by DNA-dependent protein kinase catalytic subunit (DNA-PKcs) can cut off the non-base-paired flap to expose the imbedded short microhomology (of ~4 bp). **ac** | Incompatible 3′ overhang ends are available for iterative processing until a thermodynamically stable junction is achieved through hydrogen bonding across the double-strand break junction. Artemis–DNA-PKcs mediates end resection, and DNA polymerases (Pol) add nucleotides to the ends, thereby generating short microhomologies between ends (orange). **ad** | Incompatible 5′ overhang ends can be readily trimmed by Artemis–DNA-PKcs or filled-in by DNA polymerases to generate blunt

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ends. **B** | Polymerase activity at different end configurations. **Ba** | Polymerase-μ (Polμ) and terminal deoxynucleotidyl transferase (TdT) can add nucleotides to a blunt end in a template-independent manner. **Bb** | Polλ and Polμ can fill in gaps at 3′ recessed DNA ends. **Bc** | Polλ and Polμ can add nucleotides to the blunt end in a template-dependent manner. The preferentially added nucleotides are complementary to the terminal bases at the other DNA end. **Bd** | Polλ and polμ can fill in gaps at junctions. **Be** | Polμ, TdT and Polλ can perform templated in *trans* synthesis for overhangs with short regions of terminal base pairing; that is, the polymerases can use a 3′ overhang of another DNA end as a template for nucleotide addition. Polμ and TdT have higher activity than Polλ in this context. **Bf** | Pol μ and TdT can add nucleotides to 3′ non-complementary overhangs in a template-dependent manner. **Bg** | The 3′ primer end (light blue) can slip inwards, followed by synthesis by Polμ and Polλ, leading to the generation of direct repeats, which are found at some non-homologous DNA end joining (NHEJ) repair junctions[107]. Polλ may have higher activity of generating repeats than Polμ. **Bh** | When Polμ or TdT adds nucleotides in a template-independent manner, the newly generated overhang may fold back and allow continued synthesis from the same strand end by Polμ or Polλ. The template-independent addition and then fold-back synthesis can generate inverted repeats at NHEJ junctions[15]. The orange lines represent added nucleotides.

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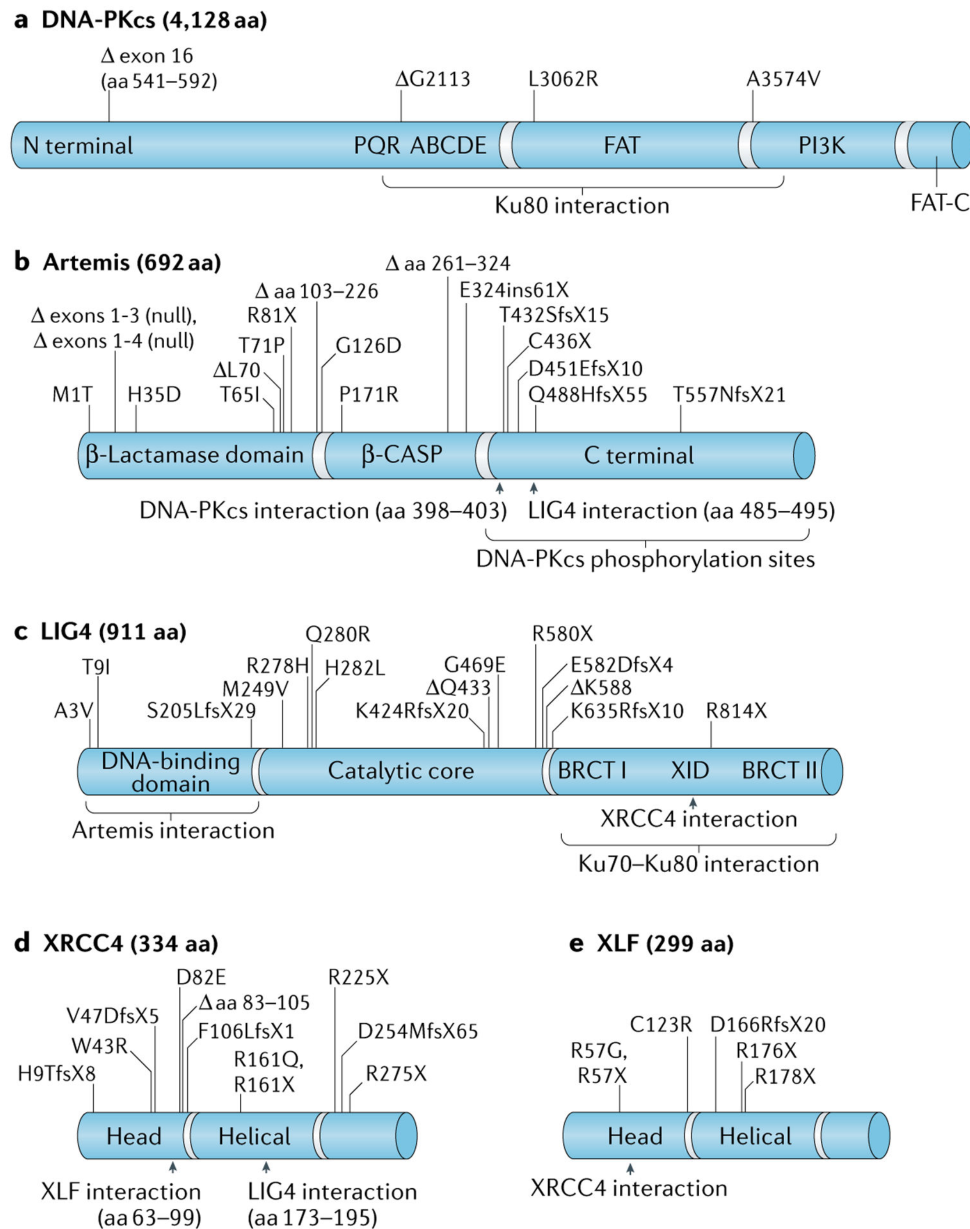

Fig. 5 |. Disease-related NHEJ hypomorphic protein variants identified in humans.

Locations of mutations identified in humans giving rise to hypomorphic non-homologous DNA end joining (NHEJ) proteins are shown. The clinical features related to these hypomorphic variants are listed in Supplementary Table 1. Additional Artemis alterations identified in humans are reported elsewhere[230]. Protein domains and their approximate positions are also shown. Blue parts represent protein domains, and grey parts represent linker regions. ‘ ’ represents a deletion, and ‘X’ denotes a stop codon. The number following ‘X’ denotes the number of amino acids (aa) from the mutation to the stop codon. β-CASP, cleavage and polyadenylation specificity factor domain; ABCDE, DNA-PKcs autophosphorylation cluster spanning residues 2609–2647; BRCT, breast cancer-associated carboxy-terminal domain; DNA-PKcs, DNA-dependent protein kinase catalytic subunit;

FAT, FRAP, ATM and TRRAP domain; FAT-C, carboxy-terminal domain of DNA-PKcs; fs, frameshift; ins, insertion; LIG4, DNA ligase 4; PI3K, phosphatidylinositol 3-kinase domain; PQR, DNA-PKcs autophosphorylation sites spanning residues 2023–2056; XID, XRCC4 interaction domain; XLF, XRCC4-like factor.

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