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The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway

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Key Words

Ku, DNA-PKcs, Artemis, XRCC4, DNA ligase IV

Abstract

Double-strand DNA breaks are common events in eukaryotic cells, and there are two major pathways for repairing them: homologous recombination (HR) and nonhomologous DNA end joining (NHEJ). The various causes of double-strand breaks (DSBs) result in a diverse chemistry of DNA ends that must be repaired. Across NHEJ evolution, the enzymes of the NHEJ pathway exhibit a remarkable degree of structural tolerance in the range of DNA end substrate configurations upon which they can act. In vertebrate cells, the nuclease, DNA polymerases, and ligase of NHEJ are the most mechanistically flexible and multifunctional enzymes in each of their classes. Unlike repair pathways for more defined lesions, NHEJ repair enzymes act iteratively, act in any order, and can function independently of one another at each of the two DNA ends being joined. NHEJ is critical not only for the repair of pathologic DSBs as in chromosomal translocations, but also for the repair of physiologic DSBs created during variable (diversity) joining [V(D)J] recombination and class switch recombination (CSR). Therefore, patients lacking normal NHEJ are not only sensitive to ionizing radiation (IR), but also severely immunodeficient.

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THE BIOLOGICAL CONTEXT OF NONHOMOLOGOUS DNA END JOINING

NHEJ: nonhomologous DNA end joining

Unlike most other DNA repair and DNA recombination pathways, nonhomologous DNA end joining (NHEJ) in prokaryotes and eukaryotes evolved along themes of mechanistic flexibility, enzyme multifunctionality, and iterative processing to achieve repair of a diverse range of substrate DNA ends at double-strand breaks (DSBs) (1–3). Except for very limited protein homology for the Ku protein in prokaryotes and eukaryotes (2), the actual nuclease, DNA polymerase, and ligase

components of NHEJ appear to have arisen independently but converged on these same mechanistic themes to handle the challenge of joining two freely diffusing ends of diverse DNA end configuration with a wide range of base or sugar oxidative damage (3).

Homology-Directed Repair versus Nonhomologous DNA End Joining

When DSBs arise in any organism, prokaryotic or eukaryotic, there are two major categories of DNA repair that can restore the duplex structure (**Figure 1**). If the organism is diploid (even if the diploidy is only transient, as in replicating bacteria or replicating haploid yeast), then homology-directed repair (HDR) can be used. The most common form of HDR is called homologous recombination (HR), which has the longest sequence homology requirements between the donor and acceptor DNA. Other forms of HDR include single-strand annealing and breakage-induced replication, and these require shorter sequence homology relative to HR (4, 5).

HR: homologous recombination

In nondividing haploid organisms or in diploid organisms that are not in S phase, a homology donor is not nearby. Hence, early in

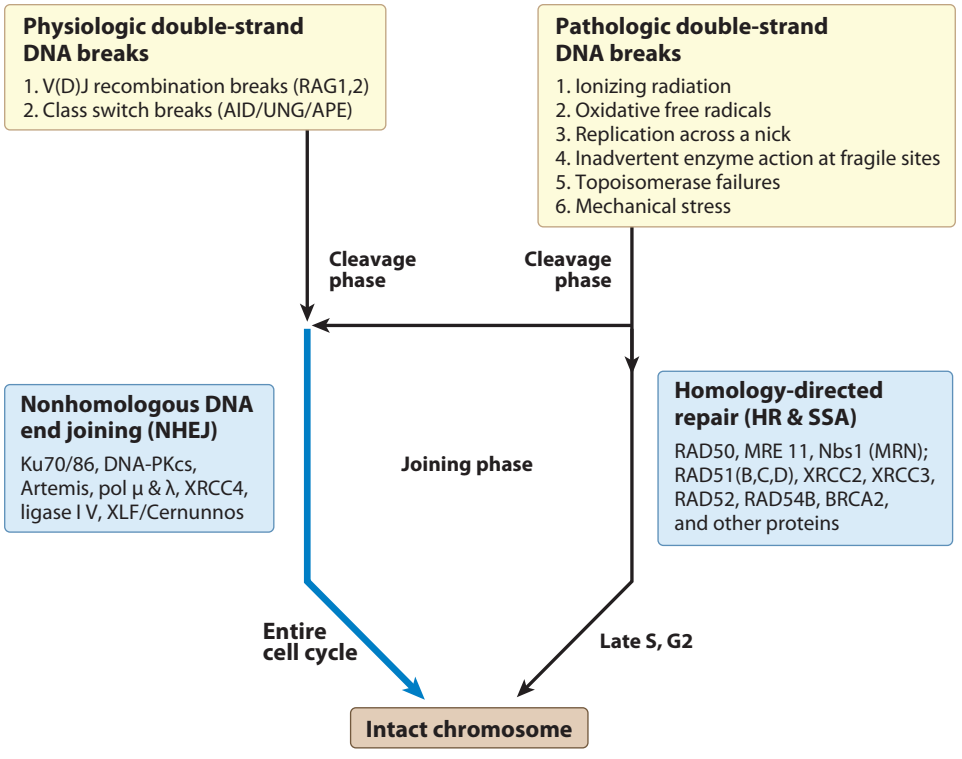

Figure 1

Causes and repair of double-strand DNA breaks. Physiologic and pathologic causes of double-strand breaks in mammalian somatic cells are listed at the top. During S and G2 phases of the cell cycle, homology-directed repair is common because the two sister chromatids are in close proximity, providing a nearby homology donor. Homology-directed repair includes homologous recombination (HR) and single-strand annealing (SSA). At any time in the cell cycle, double-strand breaks can be repaired by nonhomologous DNA end joining (NHEJ). Proteins involved in the repair pathways are listed. The NHEJ blue arrow is thicker to indicate its more frequent usage. Abbreviations for proteins listed in the figure include the following: AID, activation-induced deaminase; APE, apurinic/apyrimidinic endonuclease; pol μ, DNA polymerase μ; pol λ, DNA polymerase λ; and UNG, uracil N-glycosylase.

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Table 1 Corresponding enzymes in prokaryotic and eukaryotic NHEJ (3)

Functional component	Prokaryotes	Eukaryotes	
		Saccharomyces cerevisiae	Multicellular eukaryotes
Tool belt protein	Ku (30–40 kDa)	Ku 70/80	Ku 70/80
Polymerase	Pol domain of LigD	Pol4	Pol μ and pol λ
Nuclease	Uncertain	Rad50:Mre11:Xrs2 (FEN-1)	Artemis:DNA-PKcs
Kinase/phosphatase	Phosphoesterase domain of LigD	Tpp1 and others	PNK and others
Ligase	Ligase domain of LigD	Nej1:Lif1:Dnl4	XLF:XRCC4: DNA ligase IV

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evolution, another form of DSB repair had an opportunity to provide survival advantage, and nonhomologous DNA end joining (NHEJ) includes a set of DNA enzymes that have the mechanistic flexibility to provide such an advantage (**Table 1**) (6).

How the cell determines whether HR or NHEJ will be used to repair a break is still an active area of investigation. The HR versus NHEJ determination may be somewhat operational (7). If a homolog is not present near a DSB during the S/G2 phases, then HR cannot proceed, and NHEJ is the only option. During S phase, the sister chromatid is physically very close, thereby providing a homology donor for HR. Outside of the S/G2 phases, NHEJ is indeed the markedly preferred option. The precise molecular events, beyond issues of proximity and possible competition between Ku and RAD51 or -52, are yet to be deciphered (7–9). Recent data from *Saccharomyces cerevisiae* suggests that the DNA ligase IV complex may be key in suppressing the DNA end resection needed to initiate HR (10).

Causes and Frequencies of Double-Strand Breaks

There are an estimated 10 DSBs per day per cell; this estimate is based on metaphase chromosome and chromatid breaks in early passage primary human or mouse fibroblasts (11–13). Estimates of DSB frequency in nondividing cells are difficult to make because methods for assessing DSBs outside of metaphase are subject to even more caveats of interpretation.

In mitotic cells of multicellular eukaryotes, DSBs are all pathologic (accidental) except the specialized subset of physiologic DSBs in early lymphocytes of the vertebrate immune system (**Figure 1**). Major pathologic causes of DSBs in wild-type cells include replication across a nick, giving rise to chromatid breaks during S phase. Such DSBs are ideally repaired by HR using the nearby sister chromatid.

All of the remaining pathologic forms of DSBs are repaired primarily by NHEJ because they usually occur when there is no nearby homology donor and/or because they occur outside of S phase. These causes include reactive oxygen species (ROS) from oxidative metabolism, ionizing radiation (IR), and inadvertent action of nuclear enzymes (14).

ROS are a second major cause of DSBs (**Figure 1**). During the course of normal oxidative respiration, mitochondria convert about ~0.1% to 1% of the oxygen to superoxide (O_2^-) (15). Superoxide dismutase in the mitochondrion (SOD2) or cytosol (SOD1) can convert this to hydroxyl free radicals, which may react with DNA to cause single-strand breaks. Two closely spaced lesions of this type on antiparallel strands can cause a DSB. About 10^{22} free radicals or ROS species are produced in the human body each hour, and this represents about 10^9 ROS per cell per hour. A subset of the longer-lived ROS may enter the nucleus via the nuclear pores.

A third cause of DSBs is natural IR of the environment. This includes gamma rays and X-rays. At sea level, ~300 million IR particles per hour pass through each person. As these traverse the body, they create free radicals along

IR: ionizing radiation

their path, primarily from water. When the particle comes close to a DNA duplex, clusters of free radicals damage DNA, generating one DSB in the genome for every 25 sites of single-strand damage (16). About half of the IR that strikes each of us comes from outside the earth. The other half arises from the decay of radioactive elements, primarily metals, within the earth.

A fourth cause of DSBs is inadvertent action by nuclear enzymes on DNA. These include failures of type II topoisomerases, which transiently break both strands of the duplex. If the topoisomerase fails to rejoin the strands, then a DSB results (17). Inadvertent action by nuclear enzymes of lymphoid cells, such as the RAG complex (composed of RAG1 and -2) and activation-induced deaminase (AID) are responsible for initiating physiologic breaks for antigen receptor gene rearrangement; however, they sometimes accidentally act at off-target sites outside the antigen receptor gene loci (18). In humans, these account for about half of all of the chromosomal translocations that result in lymphoma.

Finally, physical or mechanical stress on the DNA duplex is a relevant cause of DSBs. In prokaryotes, this arises in the context of desiccation, which is quite important in nature (19). In eukaryotes, telomere failures can result in chromosomal fusions that have two centromeres, and this results in physical stress by the mitotic spindle (breakage/fusion/bridge cycles) with DSBs (20).

In addition to the above for mitotic cells, meiotic cells have an additional source of DSBs, which is physiologic and is caused by an enzyme called Spo11, a topoisomerase II-like enzyme (21). Spo11 creates DSBs to generate crossovers between homologs during meiotic prophase I. These events are resolved by HR. Therefore, NHEJ is not relevant to Spo11 breaks. Interestingly, it is not clear that NHEJ occurs in vertebrate meiotic cells because one group reports the lack of Ku70 in spermatogonia (22). Human spermatogonia remain in meiotic prophase I for about 3 weeks, and human eggs remain in meiotic prophase I for 12 to 50 years; hence, these cells can rely on HR during these long periods. Given the error-prone nature of NHEJ (see below), reliance on HR may be one way to minimize alterations to the germ line at frequencies that might be deleterious to a population.

V(D)J: variable (diversity) joining

MECHANISM OF NONHOMOLOGOUS DNA END JOINING

The enzymes of NHEJ are able to function on a diverse range of DNA ends as substrates, as discussed in detail in the following sections.

Structural Diversity of Double-Strand Break DNA Ends

Perhaps the most intriguing aspect of NHEJ is the diversity of substrates that it can accept and convert to joined products. This demands a remarkable level of mechanistic flexibility at the level of protein-substrate interaction and is unparalleled in most other biochemical processes. Though we have substantial amounts of information on the DNA end configurations at DSBs, there are limitations to the information because of the diverse manner in which IR and ROS interact with DNA. Therefore, we know the most about the diversity of physiologic DSBs, specifically V(D)J recombination, because we know where the relevant enzymes initiate the cutting of the two DNA strands. In V(D)J recombination, we can examine many NHEJ outcomes from the same starting substrates. We can also vary the sequence of the two DNA ends being joined. All of these various overhangs are joined in vivo at about the same efficiency, regardless of sequence. Within this range of overhang variation then, NHEJ can accept a wide variety of overhang length, DNA end sequence, and DNA end chemistry.

Overview of the Proteins and Mechanism of Vertebrate Nonhomologous DNA End Joining

Like most DNA repair processes, NHEJ requires a nuclease to resect damaged DNA,

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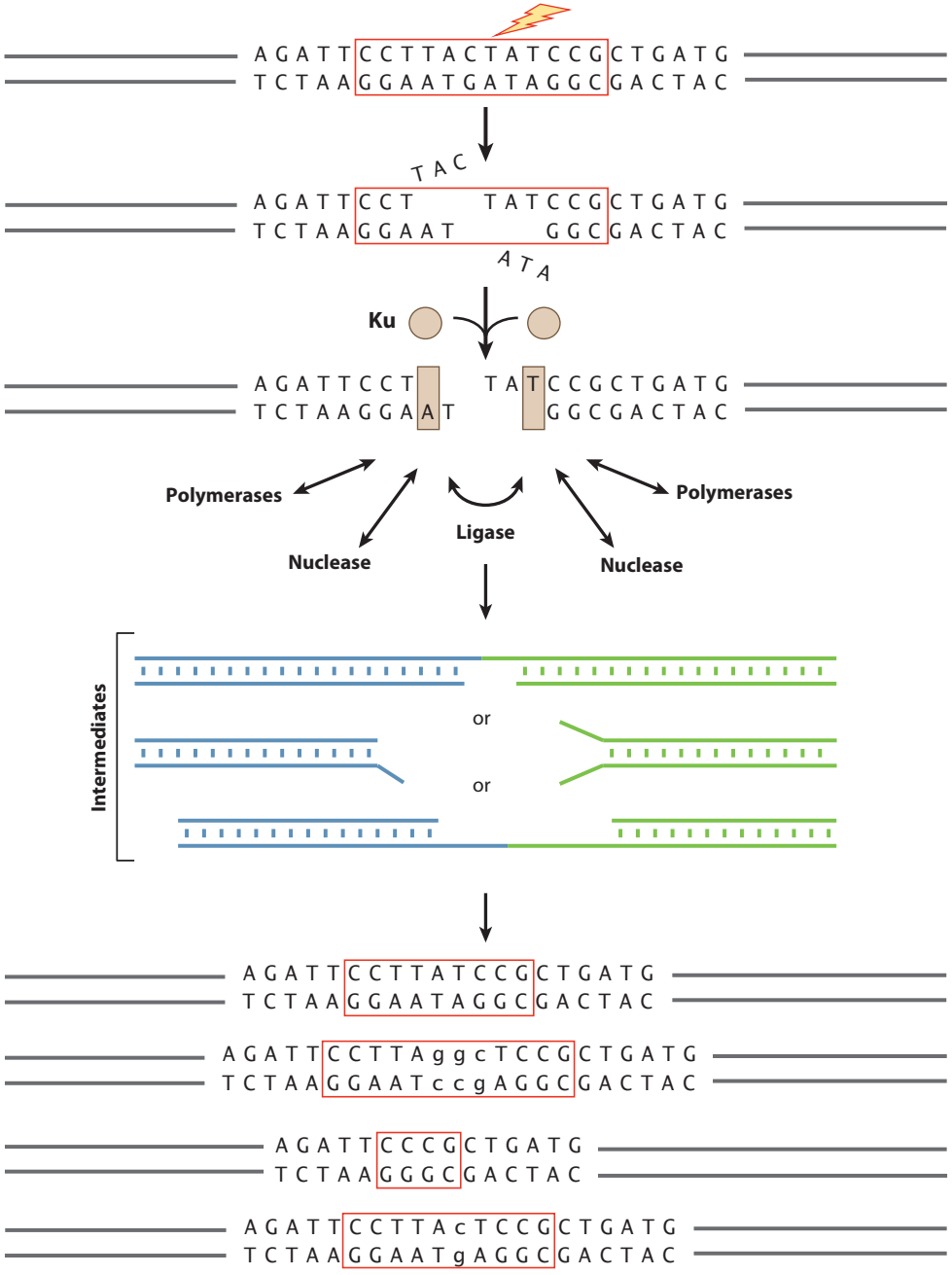
DNA polymerases to fill in new DNA, and a ligase to restore integrity to the DNA strands (**Figure 2**). Functional correspondence between the NHEJ proteins of prokaryotes, yeast (along with plants and invertebrates), and vertebrates can be inferred (**Table 1**). Prokaryotic NHEJ has been reviewed recently (23), and comparisons between prokaryotes and eukaryotes have been made (3). NHEJ in yeast, which appears to be similar for plants and



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invertebrates, has been thoroughly reviewed as well (24, 25). Hence, the discussion here focuses on NHEJ in vertebrates, with appropriate comparisons to prokaryotic and yeast NHEJ.

When a DSB arises in vertebrates, it is thought that Ku is the first protein to bind on the basis of its abundance (estimated at ~400,000 molecules per cell) and its strong equilibrium dissociation constant (~10^{-9} M) for duplex DNA ends of any configuration (**Figures 3** and **4*a***) (26–29). Ku is a toroidal protein because of its crystal structure (30). Ku bound to a DNA end can be considered as a Ku:DNA complex, which serves as a node at which the nuclease, polymerases, and ligase of NHEJ can dock (31). One can think of Ku as a tool belt protein, similar to PCNA in DNA replication, where many proteins can dock. At a DSB, there are two DNA ends. Hence, it is presumed that there is a Ku:DNA complex at each of the two DNA ends being joined, thereby permitting each DNA end to be modified in preparation for joining.

Each Ku:DNA end complex can recruit the nuclease, polymerase, and ligase activities in any order (31, 32). This flexibility is the basis for the diverse array of outcomes that can arise from identical starting ends. The processing of the two DNA ends may transiently terminate when there is some small extent of annealing between the two DNA ends. The processing may permanently terminate when one or both strands of the left and right duplexes are ligated.

Ku likely changes conformation when bound to a DNA end versus when Ku is in free solution. The basis for this inference is that Ku does not form stable complexes with DNA-PKcs in the absence of DNA ends (33), and the same appears to apply for its interactions with DNA pols μ and λ and with XRCC4:DNA ligase IV (34, 35). The crystal structure for Ku lacks the C-terminal 19 kDa [167 amino acids (aa)] of Ku86 (an important region of interaction with DNA-PKcs and other proteins), and this may be a region for conformational change upon Ku binding to DNA (36).

The Artemis:DNA-PKcs complex has a diverse array of nuclease activities, including 5′ endonuclease activity, 3′ endonuclease

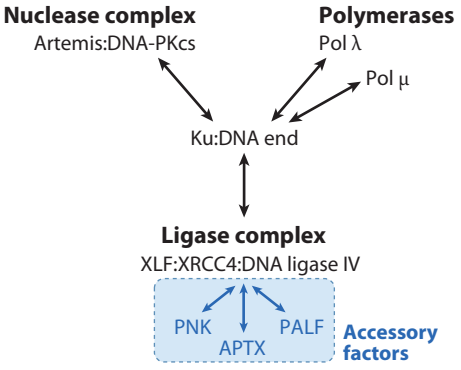

Figure 3

Interactions between nonhomologous DNA end-joining (NHEJ) proteins. Physical interactions between NHEJ components are summarized. In addition, interactions between XRCC4 and DNA-PKcs are discussed in the text, as are functional interactions. Abbreviations: APTX, aprataxin; PALF, PNK-APTX-like factor; PNK, polynucleotide kinase; XLF, XRCC4-like factor.

Figure 2

General steps of nonhomologous DNA end joining (NHEJ). The lightning arrow indicates ionizing radiation (IR), a reactive oxygen species (ROS), or an enzymatic cause of a DSB. Ku binding to the DNA ends at a double-strand breaks (DSBs) improves binding by nuclease, DNA polymerase, and ligase components. Note that Ku is thought to change conformation upon binding to the DNA end, as depicted by its shape change from a sphere to a rectangle. Flexibility in the loading of these enzymatic components, the option to load repeatedly (iteratively), and independent processing of the two DNA ends all permit mechanistic flexibility for the NHEJ process. This mechanistic flexibility is essential to permit NHEJ to handle a very diverse array of DSB end configurations and to join them. In addition to the overall mechanistic flexibility, each component exhibits enzymatic flexibility and multifunctionality, as discussed in the text. The figure shows that there are many alternative intermediates in the joining process (*middle*). These intermediates are reflected in a diverse DNA sequences at the junction of the joining process (*bottom*).

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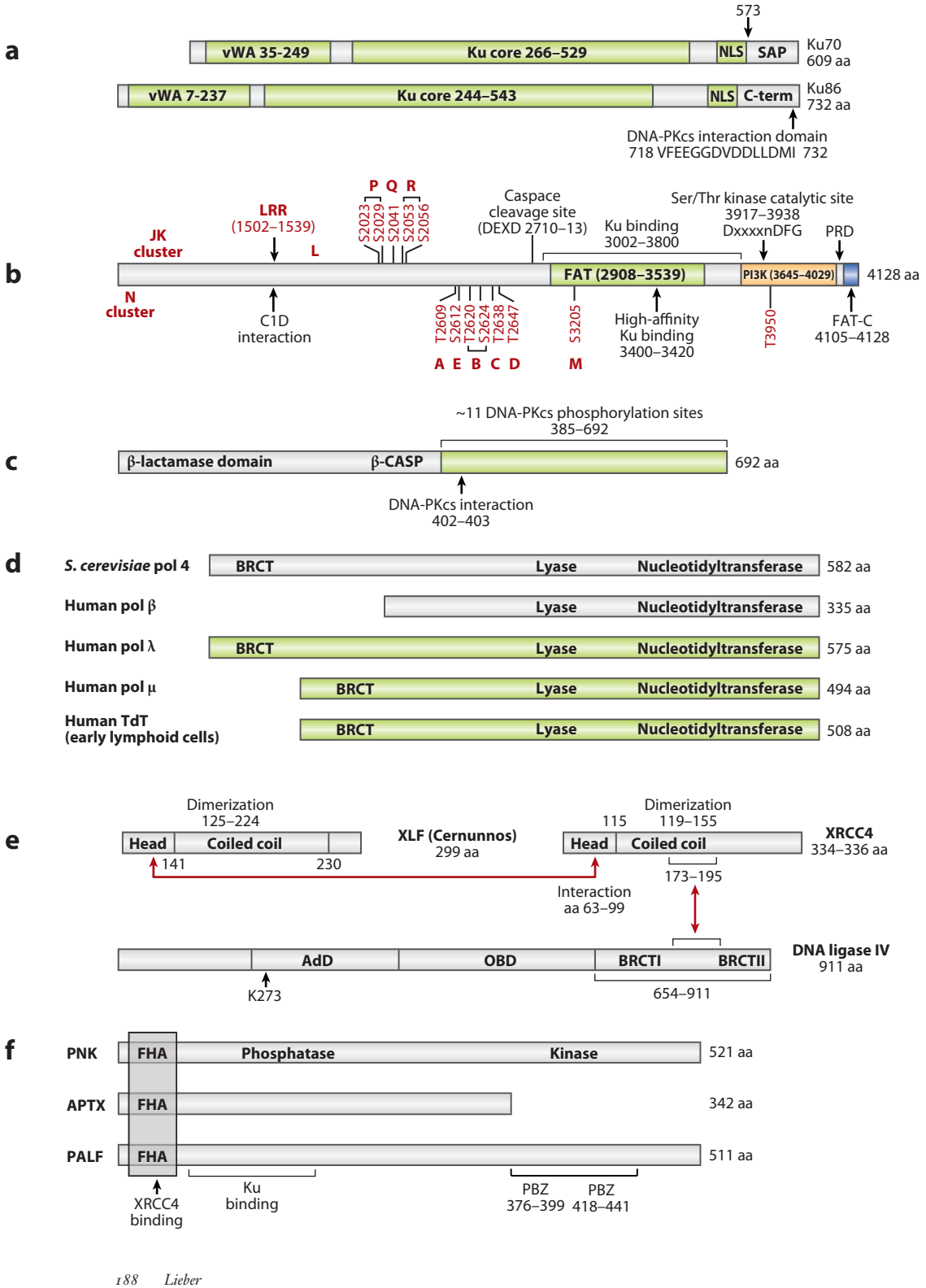

activity, and hairpin opening activity, in addition to an apparent 5′ exonuclease activity of Artemis alone (**Figure 4*b*,*c*** and **Supplemental Figure 1**. Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org.**) (37). The Artemis:DNA-PKcs complex is able to endonucleolytically cut a variety of types of damaged DNA overhangs (38, 39). Hence, there is no obvious need for additional nucleases, although the 3′ exonuclease of PNK-APTX-like factor (PALF or APLF) and others are possibilities (see the Future Issues section, below).

Pols μ and λ are both able to bind to the Ku:DNA complexes by way of their BRCT domains located in the N-terminal portion of each polymerase (**Figure 4*d***) (32). Additional polymerases appear able to contribute when neither of these two polymerases is present (40, 41). As discussed below, pol μ is particularly well suited for functioning in NHEJ because it is capable of template-independent synthesis, in addition to template-dependent synthesis. Pol λ also has more flexibility than replicative polymerases.

A complex of XLF:XRCC4:DNA ligase IV is the most flexible ligase known, with the ability to ligate across gaps and ligate incompatible DNA ends (**Figure 4*e***) (42, 43). It can also ligate one strand when the other has a complex configuration (e.g., bearing flaps), and it can ligate single-stranded DNA (ssDNA), although with limited and substantial sequence preferences.

Therefore, the nuclease, polymerases, and ligase of NHEJ all have much greater mechanistic flexibility than their counterparts in other repair pathways. This flexibility permits these structure-specific proteins to act on a wider range of starting DNA end structures. One consequence of such flexibility in vertebrates may be the substantial diversity of junctional outcomes observed, even from identical starting ends, as discussed in the next section.

Variation in Products Even from Identical Starting Substrates

If we arbitrarily designate the two DNA ends as left and right, then the Ku bound at the left end could conceivably recruit the nuclease, and the Ku at the right DNA end might recruit the polymerase, or vice versa. It is likely that there are multiple rounds of action by the nuclease, polymerases, and ligase at the left and right ends of the DSB until the top or bottom strand is ligated. Therefore, the joining of the two ends

Figure 4

Diagrams of domains within nonhomologous DNA end-joining (NHEJ) proteins. (*a*) Ku is a heterodimer of Ku70 and 86. vWA designates von Willebrand domains. SAP designates a SAF-A/B, Acinus, and PIAS domain and may be involved in DNA binding. (*b*) DNA-PKcs autophosphorylation sites are shown in red (90, 95, 96). The function of each phosphorylation site (A-E, L, M, P-R) and the clusters (N and JK) are still under study. Adjacent phosphorylation sites that are linked by a bracket have not been functionally dissected from one another. LRR designates the leucine-rich region. The FAT-C domain is a FAT domain at the C terminus. PI3K designates the PI3 kinase domain. PRD designates the PI3K regulatory domain. (*c*) Artemis is phosphorylated by DNA-PKcs at 11 sites within the C-terminal portion (*green*) (161, 162). Amino acids 156 to 385 share conserved sequence with those metallo-β-lactamases that act on nucleic acids (163). This region has been called the β-CASP domain (metallo-β-lactamase-associated CPSF Artemis SNM1 PSO2) (164). (*d*) Polymerase X (Pol X) family. Pol μ and pol λ are generally involved in NHEJ in mammalian somatic cells. Terminal deoxynucleotidyl transferase (TdT) is only expressed in early lymphoid cells, where it participates in NHEJ primarily in the context of V(D)J recombination. (*e*) The NHEJ ligase complex consists of XLF (Cernunnos), XRCC4, and DNA ligase IV. The red arrows indicate the regions of physical interaction (118, 119). OBD in ligase IV is the oligo-binding domain, and AdB is the adenylation domain. (*f*) Polynucleotide kinase (PNK), aprataxin (APTX), and PNK-APTX-like factor (PALF, which is also called APLF) are ancillary components that bind to XRCC4 of the ligase complex. The PBZ domain appears to be important for poly-ADP ribose polymerase-1 (PARP-1) binding, for poly-ADP ribose binding, and for nuclease activity. FHA designates the forkhead-associated domain. Abbreviations: aa, amino acid; BRCTI and BRCTII, BRCA-1 C-terminal domain I and II; C-term, C terminus; DNA-PKcs, DNA-dependent protein kinase; N, N terminus; NLS, nuclear localization sequence.

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is likely to be an iterative process with multiple possible routes all leading to a joining event, but with wide variation in the precise junctional sequence of the products.

Unlike pathologic causes of DSBs, which generally cannot generate predictable pairs of starting DNA ends, V(D)J recombination always generates two hairpinned coding ends (see the section on V(D)J recombination, below, for a full discussion, including signal ends). Though these two coding ends can be opened in a few different ways, the predominant hairpin opening position is 2 nucleotides (nt) 3′ of the hairpin tip in vivo (44) and in vitro (37). Hence, the two coding ends are often 3′ overhangs with a length of 4 nt each. Assuming that NHEJ within vertebrate B cells is representative of NHEJ in other tissue cell types (these junctions can be analyzed in cells that do not express terminal transferase), several inferences can be drawn from these relatively defined starting DNA ends.

First, the amount of nucleolytic resection (loss) from each DNA end varies, usually over a range of 0 to 14 base pairs (bp); but there are less frequent examples with resection up to ~25 bp (45). The rare instances where there is a loss greater than 25 bp may represent cases where the DNA end is released prematurely from whatever factors retain the two DNA ends in some proximity (see below). In vertebrate NHEJ, a complex of Artemis:DNA-PKcs is capable of endonucleolytically resecting a wide range of DNA end configurations. In yeast, plants, and invertebrates, the MRE11/RAD50/XRS2 (MRX) complex appears to be critical for some of the DNA end resection (24). The evolutionary inception of Artemis and DNA-PKcs coincides with the inception of V(D)J recombination (the vertebrate to invertebrate transition). The MRX nuclease system and the DNA-PKcs system both rely on the same conserved C-terminal tail for protein-protein interaction, also suggesting that the Artemis:DNA-PKcs complex may have evolved to replace the MRX complex for vertebrate NHEJ (46).

Second, nucleotide addition can occur at the DNA junction, even when terminal transferase is not present. In mammals, pol μ can add in a template-independent manner under physiologic conditions (7, 8). Mammalian pol λ does not appear to add in a template-independent manner except when Mg^{2+} is replaced with Mn^{2+} (47, 48). The precise biochemical properties of Pol X family members in other eukaryotes are not as clear. Interestingly, in bacteria, the polymerase activity intrinsic to the LigD protein is capable of adding 1 nt or ribonucleotide in an entirely template-independent manner (23), perhaps reflecting convergent evolution.

Pol μ and pol λ both seem to have much greater flexibility than most polymerases during template-dependent synthesis (47, 48). The template-independent addition by pol μ would sometimes be expected to fold back on itself (42), and the resulting stem-loop structure might function as a primer/template substrate, see step 1 in **Supplemental Figure 2**. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org** (48). This may account for the observed inverted repeats at many NHEJ junctions from chromosomal translocations in humans (49, 50). Both pol μ and pol λ can slip back on their template strand (51–53), and this may permit generation of direct repeats, accounting for such events seen in vivo (54–56). Direct repeats are also often seen at NHEJ junctions from human chromosomal translocations (49, 50). The direct and inverted repeats seen at these NHEJ junctions have been termed T nucleotides, where the T stands for templated (49, 50).

Therefore, even from a relatively homogeneous set of starting DNA ends as substrates, there is substantial variation in the nucleotide resection from each end and variation in the amount of template-independent addition to the two DNA ends. These two sources of variation are the basis for the heterogeneity at the joining site.

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Mechanistic Flexibility, Iterative Processing, and Independent Enzymatic Functions as Conserved Themes

In the context of considering diverse substrates and diverse joining products, it is worth noting an additional facet of NHEJ flexibility: The proteins involved and the order of their action in NHEJ can vary at either of the two DNA ends. Each DNA end, especially when bound by Ku, is best considered as a node at which any of the NHEJ proteins can dock. If one of the polymerases arrives first at the left end, then this might enact the first step at that end. However, if the nuclease binds first at that end, then resection will occur first (32).

In addition to the theme of mechanistic flexibility, there is the theme of iterative processing of the junction (32, 38). For any given joining event, there might, for example, be only three steps involved: one each involving a nuclease, a polymerase, and then a ligase. However, in another joining scheme, there might be 10 steps with multiple appearances by each enzyme activity. Hence, each of the enzymatic components might be involved not at all, once, or many times.

Related but in addition to the theme of iterative processing is the independent function of the nuclease, polymerases, and ligase from one another and even from Ku. Each of the enzymatic activities has a substantial range and level of activity without any of the others, and even without Ku, when examined in purified biochemical systems. For example, Ku is entirely unnecessary in ligation of DNA ends by the ligase IV complex when those ends share 4 bp of terminal microhomology, but Ku is stimulatory for shorter microhomology lengths (42). Pols μ and λ are able to carry out fill-in synthesis, and pol μ does not always require Ku or XRCC4:DNA ligase IV to have terminal deoxynucleotidyl transferase (TdT)-like activity at a DNA end (42). The Artemis:DNA-PKcs complex does not require Ku or any other component to carry out its endonucleolytic functions (37). Therefore, independent function of each enzymatic activity and iterative processing and mechanistic flexibility are all noteworthy features of vertebrate NHEJ. *S. cerevisiae* NHEJ manifests mechanistic flexibility but within a narrower range of junctional outcomes than mammalian NHEJ (24, 57).

Enzymatic Revision of a Partially Completed Junction

In the context of iterative processing, the Artemis:DNA-PKcs nuclease complex is able to nick within the single-stranded portion of a gapped structure and within a bubble structure (38). Joinings where only one strand is ligated would often have a gapped configuration. Nicking of such a gap could permit nucleotides that were originally part of the arbitrarily designated left DNA end to become separated from that left end and become associated with the right DNA end. Then further nucleotide addition at the left end could separate these nucleotides from the left end. One can find potential examples of this at in vivo junctions. In other scenarios, with the flexibility of the ligase, there may be more nucleotides on the top strand than the bottom strand (42). The activated Artemis:DNA-PKcs complex can nick mismatched or bubble structures on either strand, thereby permitting additional rounds of junctional revision (38).

Terminal Microhomology Between the Initial Two DNA Ends Can Simplify the Protein Requirements

On the basis of both *S. cerevisiae* and mammalian in vivo NHEJ studies, the variation of the resulting junction is usually less when there is terminal microhomology at the ends (24, 57–60). This may reflect the involvement of fewer NHEJ proteins, and genetic studies support the view that not all of the NHEJ components are essential when the two DNA ends share terminal microhomology (60–66).

As mentioned above, when the two DNA ends happen to share 4-nt overhangs that are perfectly complementary, then the only component needed in purified biochemical systems

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is the XRCC4:DNA ligase IV (42). No nucleolytic resection or polymerase action is needed. Using purified proteins in vitro, XRCC4:ligase IV is adequate to join such a junction, and even ligase IV alone may be sufficient, all without Ku. Moreover, ligase I or III alone is sufficient for such joinings, though at a lower efficiency (42). In vivo generation of defined-DNA end configurations at DSBs is not simple, but there are two approaches that have been used. In *S. cerevisiae*, short oligonucleotide duplexes can be ligated onto the DNA ends of a linear plasmid, and then these can be transfected into cells (57). One can then harvest the joined circular molecules for analysis. On the basis of this result, it is clear that the joining dependence is simplified when there is terminal microhomology at the DNA ends. A second system for generating defined DNA ends is V(D)J recombination, as mentioned above. These ends are not as precisely defined as in the yeast system (because the precise DNA end configuration depends on how hairpin intermediates are opened), but there is the advantage that the ends are actually generated inside the nucleus. In V(D)J recombination, the coding ends (defined in **Supplemental Figure 3**. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**) are usually configured with a 4-nt 3′ overhang. If the DNA ends are chosen to be complementary, then the dependence of the V(D)J recombination on Ku can be very minimal (60). However, NHEJ repairs such ends so as to align the microhomology in a disportionate fraction of the joins (58, 67).

Terminal Microhomology Can Bias the Diversity of Joining Outcomes, but Microhomology Is Not Essential

One of the strengths of NHEJ is that microhomology does not appear to be essential in mammalian cells (58, 67). The joining of incompatible DNA ends may be a key selective advantage that drove further evolutionary development of NHEJ in higher eukaryotes. The fact that some of this evolution was convergent rather than divergent further illustrates the strength of this selective advantage. Most natural DSBs generate incompatible ends with little or no microhomology within the first few nucleotides. [*S. cerevisiae* NHEJ shows mechanistically interesting differences from mammalian NHEJ insofar as yeast are very poor at blunt-end ligation and perhaps more reliant on at least one base pair of terminal microhomology (24, 25, 68).]

For mammalian NHEJ joins, the most common amount of observed terminal microhomology is 0 nt (45, 69). The next most common is 1 nt, and longer microhomologies are less common with increasing length. As mentioned above, when microhomology is present, then usage of that microhomology for a given pair of DNA ends can be dominant (57–60).

Overhangs with substantial terminal microhomology are uncommon in nature and are limited primarily to regions containing repetitive DNA. Like wild-type cells, in neoplastic cells arising in normal animals, the most common amount of terminal microhomology at NHEJ junctions is also zero. Increases in microhomology usage occur in two circumstances. First, if the experimental system being used specifically positions terminal microhomology at or near the DNA ends, then the high-microhomology outcome might be observed. Second, in animals lacking a complete wild-type NHEJ system, the NHEJ process may be slower, may show more resection, and may seek end alignments that are stabilized by more terminal microhomology (2 or 3 bp), as discussed in the next section.

Alternative Nonhomologous DNA End Joining

In experimental systems, when one or more proteins of NHEJ are mutated, the joining that occurs is said to be due to alternative NHEJ.

Ligase IV-independent joining. It has been elegantly demonstrated in murine and yeast genetic studies that end joining can occur in the absence of ligase IV (57, 69–71). Insofar as the only remaining ligase activity in the cells is due

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to ligase I in *S. cerevisiae* or ligase I or III in vertebrate cells, these joinings must be done by ligase I or III. Most of these joinings rely more heavily on the use of terminal microhomology than NHEJ in wild-type cells. In wild-type cells, plots of end-joining frequency versus microhomology length show a peak at 0 nt of microhomology and decline for increasing lengths (69). But for joinings without ligase IV, the peak is at 2.5 bp, and the frequency declines on both sides of this peak (69–71).

In biochemical systems using purified NHEJ proteins, it has been shown that human ligase I and III are able to join DNA ends that are not fully compatible (e.g., joining across gaps in the ligated strand), although this is still substantially less efficient than joining by the XRCC4:ligase IV complex (42, 43). Though relatively inefficient, this joining by ligase I or III is somewhat more efficient with two or more base pairs of terminal microhomology to stabilize the ends. Therefore, in the absence of the ligase IV complex, it may not be surprising that the peak microhomology usage changes from zero to between 2 and 3 bp.

The in vivo joining efficiency by mammalian ligase IV relative to ligase I and III is difficult to measure. Two measurements have been done in murine cells in which the joining occurred at DNA ends that have some increased opportunities for terminal microhomology, the class switch recombination (CSR) sequences (see below for explanation of CSR). In one study, cells lacking ligase IV are removed from mice and stimulated in culture to undergo CSR (70). Measurements of switch recombination can be done as early as 60 h after stimulation. In this case, end joining without ligase IV is reduced only 2.5-fold. In another case, a murine cell line was used to make the genetic knockout, and measurements could be done as early as 24 h, at which time the joining without ligase IV was reduced about ninefold (69). In both cases, the joining is almost certainly done by ligase I or III. The latter study suggests that the joining by ligase I or III is substantially less efficient at early times. In both studies, given sufficient time, the joining by ligase I or III improves to about half that of the wild-type cells (where nearly all joining is likely, though not proven, to be the result of ligase IV). Also, in both studies, the joining in wild-type cells was much less dependent on terminal microhomology than joining in the ligase IV knockout cells. One reasonable interpretation of these two studies is that ligase IV is more efficient (and perhaps faster) at joining incompatible DNA ends in vivo but that ligase I or III can join ends at a lower efficiency, especially when terminal microhomology can stabilize the DNA ends.

In *S. cerevisiae*, end joining can occur in the absence of ligase IV, but it is at least 10-fold less efficient (24). Moreover, when the joining does occur, it tends to use microhomology (usually >4 bp) that is longer and more internal to the two DNA termini than is seen for wild-type yeast (57).

Ku-independent joining. Ku-independent end joining also occurs in both *S. cerevisiae* and mammalian cells. In yeast, such events can even be as efficient as end joining in the corresponding wild-type cells (24, 57). For ligase IV mutants in yeast, the joining relies on longer microhomology (usually >4 bp) that is more internal to the two DNA ends. Ku-independent end joining also can be seen in mammalian cells (60). Even in vertebrate V(D)J recombination, when the two DNA ends share 4 bp of terminal microhomology, the dependence on Ku for joining efficiency can be small (2.5-fold) (60). This indicates that terminal microhomology can substitute for the presence of Ku. We do not know with certainty what Ku-independent joining means mechanistically, but one possibility is that the ligase IV complex normally holds the DNA ends and that Ku stabilizes the ligase IV complex. But when Ku is absent, terminal microhomology may provide some of this stability, consistent with observations in biochemical systems (42).

Zero microhomology joins in the absence of ligase IV. For some ends joined in the absence of ligase IV, no microhomology is obvious (66). This raises the question whether ligase I

CSR: class switch recombination

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or III can join ends with no terminal microhomology. XRCC4:ligase IV can ligate blunt ends (72), and ligase III is also able to do this at low efficiencies (35). All three ligases can do so when macromolecular volume excluders are present (35). Nevertheless, blunt-end ligation is much less efficient for all three ligases.

Another explanation of such events is that they involve template-independent synthesis by the Pol X family members (42). As discussed, in mammalian cells, pol μ and pol λ participate in NHEJ (42, 73, 74), as does Pol4 in *S. cerevisiae* (75). In Mn^{2+} buffers, both pol μ and pol λ can add nucleotides independently of a template, and in the more physiologic Mg^{2+} buffers, pol μ still shows robust template-independent addition. Such template-independent activity could permit additions to DNA ends that provide microhomology with another end; because that addition would be random, it would not have been scored as microhomology. One could consider such inapparent microhomology as polymerase-generated microhomology (also called invisible or occult microhomology, see below). This type of microhomology would not have been present in the two original DNA end sequences, and in that sense, it can be regarded as polymerase-generated microhomology.

In addition to template-independent synthesis by pol μ (which pol μ exhibits alone or in the context of other NHEJ proteins), pol μ together with Ku and XRCC4:ligase IV can synthesize across a discontinuous template, and this would also generate microhomology. However, this mechanism requires that the DNA end providing the template have a 3′ overhang to permit the polymerase to extend into that end. Hence, only a subset of DNA ends could be handled in this manner.

The ratio of template-independent versus template-dependent synthesis by pol μ at NHEJ junctions in such cases is not entirely clear, but both mechanisms occur under physiologic conditions in biochemical systems, and there is clearly some evidence for the template-independent pol μ addition within mammalian cells (41).

Nomenclature. In all organisms in which there is NHEJ, there are examples of DNA end joining in the absence of the major NHEJ ligase of that organism (76); even in mycobacteria, LigC can function in NHEJ when LigD is absent (77). Given that the ligase is often regarded as the signature enzymatic requirement of NHEJ, these joining events have been proposed to be the result of alternative NHEJ, or backup NHEJ. As mentioned above for most of these end joinings, there is substantial terminal microhomology. Hence, in *S. cerevisiae*, this joining has also been called microhomology-mediated end joining, but it is essentially an alternative NHEJ.

For eukaryotic end joining, one reasonable nomenclature is to use NHEJ as the general term and to simply note the exceptions, as for example, ligase IV-independent NHEJ, Ku-independent NHEJ, or DNA-PKcs-independent NHEJ (i.e., X-independent NHEJ, where X is the omitted protein). Until a specific pathway is delineated, this is a practical solution. It is quite conceivable (even likely) that ligase IV-independent NHEJ is merely NHEJ in which ligase I or III completes the ligation at a somewhat lower efficiency than ligase IV.

Evolutionary Comparisons of Nonhomologous DNA End Joining

Initially, NHEJ was thought to be restricted to eukaryotes because the best-studied prokaryote, *Escherichia coli*, cannot recircularize linear plasmids. However, when bioinformatists discovered a distantly diverged Ku-like gene in prokaryotic genomes, the existence of a similar NHEJ pathway in bacteria became clear (2, 78, 79). The bacterial Ku homolog appears to form a homodimer with a structure similar to the ring-shaped eukaryotic Ku heterodimer (80). The gene for an ATP-dependent ligase named LigD was typically found to be adjacent to the gene for Ku on the bacterial chromosome (2, 78). This linkage between Ku and an ATP-dependent ligase prompted extensive studies, which later defined a bacterial NHEJ pathway. In most bacterial species, unlike the eukaryotic

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NHEJ ligase IV, LigD is a multidomain protein that contains three components within a single polypeptide: a polymerase domain, a phosphoesterase domain, and a ligase domain (23).

Why do not all bacteria have an NHEJ pathway? Bacterial NHEJ is nonessential under conditions of rapid proliferation because HR is active and a duplicate genome is present to provide homology donors (23, 81). Those bacteria that have the NHEJ pathway spend much of their life cycle in stationary phase, at which point HR is not available for DSB repair because of a lack of homology donors. In addition, desiccation and dry heat are two naturally occurring physical processes in nature that produce substantial numbers of DSBs in bacteria. Therefore, bacterial Ku and LigD are present in bacterial species that often form endospores because NHEJ is important for repair of DSB arising during long periods of sporulation.

INDIVIDUAL PROTEINS OF VERTEBRATE NONHOMOLOGOUS DNA END JOINING

Each of the individual NHEJ proteins carries an interesting detailed functional and structural literature, and more detailed reviews of each individual component are cited.

Ku

Ku was named on the basis of protein gel mobilities (actually 70 kDa and 83 kDa) of an autoantigenic protein from a scleroderma patient with the initials K.U. Ku86 is also called Ku80. The toroidal shape of Ku is consistent with studies showing that purified Ku can bind at DNA ends, and yet Ku can also slide internally at higher Ku concentrations (82). Ku can only load and unload at DNA ends. When linear molecules bearing Ku are circularized, the Ku proteins are trapped on the circular DNA. A minimal footprint size for Ku is ~14 bp at a DNA end (83). The key aspects of Ku in NHEJ have been discussed above, and the reader is referred to a detailed review about Ku for additional information (see Reference 84).

DNA-PKcs

DNA-PKcs has a molecular weight of 469 kDa and has 4128 aa. It is the largest protein kinase in biology, and the only one that is specifically activated by binding to duplex DNA ends of a wide variety of end configurations (33, 85–87). DNA-PKcs alone has an equilibrium dissociation constant of 3×10^{-9} M for blunt DNA ends, and this tightens to 3×10^{-11} M when Ku is also present at the end (88). Once bound, DNA-PKcs acquires serine and threonine kinase activity (89). But its initial phosphorylation target seems to be itself, with more than 15 autophosphorylation sites and probably with an equal number not yet defined (90). In addition to the relationship with Artemis discussed above, DNA-PKcs interacts with XRCC4 and phosphorylates a very long list of proteins in vitro (26, 91). In vivo evidence for functional effects of the additional protein phosphorylation targets is limited.

The best current structural information concerning DNA-PKcs alone is at 7-Å resolution by cryoelectron microscopy (cryo-EM) (92). At this resolution, α-helices are resolved, but this cryo-EM structure only contains a fraction of the total number of α-helical densities expected and therefore could not definitively reveal which portions of the structure are related to the primary amino acid sequence of DNA-PKcs. The "crown" in that structure is thought to contain the FAT domain and possibly parts of the kinase domain (**Figure 4*b***) (92). The base in that structure is the same as "proximal claws 1 and 2" in a cryo-EM structure of Ku:DNA-PKcs:DNA by another group and was shown to contain HEAT-like repeats at 7-Å resolution (93). In the Ku:DNA-PKcs:DNA and DNA-PKcs:DNA structures, the path of the duplex DNA is not entirely certain, and it is not clear which side of Ku is bound to DNA-PKcs (93, 94). The position of the C-terminal portion of Ku when bound to DNA-PKcs is also not determined, which is important because this

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interaction activates DNA-PKcs and is defined at the primary sequence level (46). Continued work using cryo-EM and other structural methods will undoubtedly be of great value.

It is not clear whether DNA-PKcs remains bound to the DNA ends throughout all processing steps of NHEJ (31, 95). Phosphorylation at the ABCDE cluster appears to increase the ability of other proteins, such as ligases, to gain access to the DNA ends, suggesting that DNA-PKcs may dissociate more readily after autophosphorylation at these sites (90, 95, 96).

DNA-PKcs interaction with other proteins is also important. As mentioned above, DNA-PKcs is critical for the endonucleolytic activities of Artemis (31, 37, 39, 97, 98). Activated DNA-PKcs stimulates the ligase activity of XRCC4:DNA ligase IV (90, 95, 96). Interestingly, the presence of XRCC4:DNA ligase IV stimulates the autophosphorylation activity of DNA-PKcs (96). Therefore, DNA-PKcs may be critical for the nucleolytic step, but also stimulatory for the ligation step.

Artemis

The Artemis:DNA-PKcs complex has 5′ endonuclease activity with a preference to nick a 5′ overhang so as to leave a blunt duplex end (37). The Artemis:DNA-PKcs complex also has 3′ endonuclease activity with a preference to nick a 3′ overhang so as to leave a 4-nt 3′ overhang. In addition, the Artemis:DNA-PKcs complex has the ability to nick perfect DNA hairpins at a position that is 2 nt past the tip. These three seemingly diverse endonucleolytic activities at single- to double-strand DNA transitions are similar to one another if one infers the following model for binding of the Artemis:DNA-PKcs complex to DNA (**Supplemental Figure 1**) (37). The complex appears to localize to a 4-nt stretch of ssDNA adjacent to a single-/double-strand transition and then nick on the 3′ side of that ssDNA 4-nt region. This would explain why 5′ overhangs are preferably removed to generate a blunt DNA end, but 3′ overhangs are nicked so as to preferably leave a 4-nt 3′ overhang. Moreover, it explains why a hairpin is nicked not at the tip but 2 nt 3′ of the tip (37). In perfect DNA hairpins, the last 2 bp do not form well, which means the tip is actually similar in many ways to a 4-nt single-stranded loop. Artemis nicks the hairpin on the 3′ side of that loop. The opened hairpin then becomes a 3′ overhang of 4 nt.

In V(D)J recombination, null mutants of DNA-PKcs and of Artemis are very similar (63, 99–101). Both fail to open the DNA hairpins, but the signal ends are joined. Biochemically, when a purified complex of Artemis:DNA-PKcs binds to an individual DNA hairpin molecule, that hairpin can activate the kinase activity of that DNA-PKcs protein (in *cis*) to phosphorylate itself and the bound Artemis within the C-terminal portion (96, 102). With respect to hairpin opening and its other endonucleolytic activities, Artemis:DNA-PKcs functions as if it were a heterodimer in which mutation of either subunit results in the failure of DNA end processing. A recent DNA-PKcs point mutation in a patient supports that view (103), as does a murine knockin model that recreates a truncation mutant of Artemis that removes the C-terminal portion where the sites of DNA-PKcs phosphorylation are located.

Some DNA ends containing oxidative damage to the bases or sugars require nuclease action to remove the damaged nucleotides. In these cases, involvement of Artemis:DNA-PKcs appears critical (39, 98).

Polymerase X Family

Three polymerases of the Pol X family can participate in NHEJ.

Polymerase μ. Pol μ has several remarkable activities as a polymerase under physiologic buffer conditions. First, it can carry out template-dependent synthesis with dNTP and rNTP, and it has substantial template-independent synthesis capability, like TdT (104). No other higher eukaryotic polymerase has this range of activities. The ability to add rNTP may be important for NHEJ during G1, when dNTP levels are low, but rNTP levels

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are high (74). Incorporation of U into the junction might then mark the junction for possible revision using uracil glycosylases at a later point in time. [The highly homologous Pol4 of *S. cerevisiae* also efficiently incorporates rNTPs (105). Interestingly, the bacterial polymerase for NHEJ (part of LigD) has the ability to incorporate ribonucleotides as well (23).] Second, like many error-prone polymerases, pol μ can slip on the template strand (48, 51, 52). Third, and as mentioned above, pol μ, when together with Ku and XRCC4:DNA ligase IV, can polymerize across a discontinuous template strand, essentially crossing from one DNA end to another (106, 107). Fourth, and also mentioned previously, pol μ has template-independent activity, which pol μ exhibits whether alone or together with Ku and XRCC4:DNA ligase IV (42).

Both the template-independent and the discontinous template polymerase activities are likely to be of great importance in the joining of two incompatible DNA ends. For example, in the case of two blunt DNA ends, the TdT-like activity of pol μ allows pol μ to add random nucleotides to each end. As soon as the resulting short 3′ overhangs share even 1 nt of complementarity (polymerase-generated microhomology), then ligation is much more efficient (42). In contrast, in the mechanism where pol μ (with Ku and XRCC4:ligase IV present) crosses from one DNA end to the other (template-dependent synthesis across a discontinuous template strand), the duplex end onto which the new synthesis extends must be a 3′ overhang to permit such extension by the polymerase (107). Hence, there are two mechanisms by which pol μ can create microhomology during the joining process (and these reaction intermediates would not be scored as microhomology events merely on basis of the final DNA sequence of the junctional product).

Structural studies of pol μ, TdT, and pol λ are defining the basis for the intriguing differences between these three highly related DNA polymerases (104). A region called loop 1 (and other positions, such as H329) is important for substituting for the template strand as TdT (always) and pol μ (sometimes) polymerize in their template-indepdendent mode (108, 109). Importantly, the crystal structures are on single-strand break DNA, and therefore, we do not know how these enzymes configure on DSBs.

Polymerase λ. Mouse in vivo systems, crude extract NHEJ studies, and purified NHEJ systems support a role for pol λ in NHEJ (41, 73, 110). Pol λ functions primarily in a standard template-dependent manner in Mg^{2+} buffers, but it has template-independent activity in Mn^{2+} (48, 104). The lyase domain in pol λ is functional, whereas the ones in pol μ and TdT do not appear to be functional. This permits pol λ to function after action by a glycosylase to remove a damaged base.

Terminal deoxynucleotidyl transferase. Terminal deoxynucleotidyl transferase (TdT or terminal transferase) is only expressed in pro-B/pre-B and pro-T/pre-T stages of lymphoid differentiation (111). Like the other two Pol Xs of NHEJ, TdT has an N-terminal BRCT domain. (Pol β is the only Pol X that is not involved in NHEJ, and it lacks any BRCT domain.) TdT only adds in a template-independent manner, consistent with a different loop 1 from pols μ and λ (104). TdT prefers to stack the incoming dNTP onto the base at the 3′ OH, accounting for its tendency to add runs of purines or runs of pyrimidines (45). TdT also has a lower K_m for dGTP, and this also biases its template-independent synthesis in vitro and in vivo (111, 112).

XLF, XRCC4, and DNA Ligase IV

DNA ligase IV (also called ligase IV or DNL4) is mechanistically flexible. In the absence of XRCC4, DNA ligase IV appears to still be capable of ligating not only nicks, but even compatible (4-nt overhang) ends of duplex DNA (72). With XRCC4, ligase IV is able to ligate ends that share 2 bp of microhomology and have 1-nt

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gaps, but addition of Ku improves this 10-fold (42). When Ku is present, XRCC4:DNA ligase IV is able to ligate even incompatible DNA ends at low efficiency (42). When XLF is also added, then XLF:XRCC4:DNA ligase IV, in the presence of Ku, can ligate incompatible DNA ends much more efficiently (43, 113).

Even 1 nt of terminal microhomology markedly increases the efficiency of ligation by Ku plus XRCC4:DNA ligase IV (43, 113), but some junctions formed within cells have no apparent microhomology (45). These could be cases where Ku plus XLF:XRCC4:DNA ligase IV ligate incompatible DNA ends or blunt ends. As mentioned, pol μ may add nucleotides either without a template or across a discontinuous template strand from the left to the right DNA end, and either of these mechanisms would not be scored as use of microhomology upon inspection of the sequence of the joined product junction (polymerase-generated microhomology). DNA ligase IV is predominantly preadenylated as it is purified from crude extracts. The reader is referred to Reference 114 for more details.

XRCC4 and XLF (Cernunnos). XRCC4 can tetramerize by itself, but it is unclear what function this serves (115). The crystal structure demonstrates a globular head domain and a coiled-coil C terminus when it forms a dimer (116, 117).

The crystal structure of XLF (Cernunnos) suggests a structure similar to XRCC4, with a globular head domain and a coiled-coil C terminus, where multimerization occurs (118, 119). When XLF is missing in humans, patients are IR sensitive and lack V(D)J recombination (120, 121). In mice, the IR defect is the same as in humans, but the V(D)J recombination defect is less severe in pre-B cells and yet is severe in mouse embryonic fibroblasts (when given exogenous RAGs) from the same mice (122). Considering the biochemical role of XLF in the joining of incompatible DNA ends, it has been suggested that TdT in the pre-B cells can provide "occult" or polymerase-generated microhomology, making joining less reliant on XLF, and this is a reasonable explanation of the data thus far (122).

Complexes of XLF, XRCC4, and DNA ligase IV and interactions with other NHEJ components. The interactions between XLF, XRCC4, and DNA ligase IV have been studied genetically and biochemically (120, 121, 123, 124). Gel filtration studies of XLF, XRCC4, and DNA ligase IV are most consistent with a stoichiometry of 2 XLF, 2 XRCC4, and 1 ligase IV (120). Complexes of XRCC4 and ligase IV are most consistent with a stoichiometry of 2 XRCC4 and 1 DNA ligase IV (115, 117). Further functional and structural work on the ligase complex will be of great value.

Both for *S. cerevisiae* and in mammalian purified proteins, Ku is able to improve the binding of XRCC4:DNA ligase IV at DNA ends. This interaction requires both Ku70 and 86 and the first BRCT domain within the C-terminal portion of ligase IV (aa 644 to 748) (91). The presence of DNA-PKcs enhances this complex formation, perhaps through interactions with XRCC4 (125–127). XRCC4:DNA ligase IV is able to stimulate DNA-PKcs kinase activity (96). The ligase complex also stimulates the pol μ and λ activities in the context of Ku (96). All of these findings suggest that the NHEJ components, although capable of acting independently, also evolved to function in a manner that is synergistic when in close proximity.

Polynucleotide Kinase, Aprataxin, and PNK-APTX-Like Factor

Polynucleotide kinase (PNK), aprataxin (APTX) and PALF (also called APLF) all interact with XRCC4 (**Figures 2** and **4*f***). PNK and XRCC4 form a complex via the PNK forkhead-associated (FHA) domain, but only after the CK2 kinase phosphorylates XRCC4 (127a). This same interaction occurs between PALF and XRCC4 as well as between APTX and XRCC4.

Polynucleotide kinase. For pathologic breaks caused by IR or free radicals, PNK plays

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an important role in several ways that illustrate a corollary theme of NHEJ: one of enzymatic multifunctionality (128–130). Mammalian PNK is both a kinase and a phosphatase. PNK has a kinase domain for adding a phosphate to a 5′ OH. PNK has a phosphatase domain that is important for removing 3′ phosphate groups, which can remain after some oxidative damage or partial processing (or after NIELS 1 or 2 remove an abasic sugar, leaving a 3′ phosphate group). Interestingly, the short 3′ overhang that the Artemis:DNA-PKcs complex prefers to leave at long 3′ overhangs represents an ideal substrate for PNK to add a 5′ phosphate at a recessed 5′ OH.

Removal of 3′ phosphoglycolate groups. Oxidative damage often causes breaks that leave a 3′ phosphoglycolate group, and these can be removed in either of two major ways. First, Artemis:DNA-PKcs can remove such groups using its 3′ endonucleolytic activity (39, 98). Second, 3′ phosphoglycolates can be converted to 3′ phosphate by tyrosyl DNA phosphodiesterase 1, whose major role is the removal of tyrosyl-phosphate linkages that arise when topoisomerases fail to religate transient DNA single-strand break reaction intermediates. Then PNK can remove the 3′ phosphate group.

Aprataxin. Aprataxin (APTX) is important in deadenylation of aborted ligation products in which an AMP group is left at the 5′ end of a nick or DSB owing to a failed ligation reaction (131, 132).

PNK-APTX-like factor. PALF and APLF are the same protein (511 aa, 57 kDa). The PALF designation stands for PNK and APTX-like FHA protein (133). APLF stands for aprataxin- and PNK-like factor (134, 135). Previously, it was also called C2orf13. PALF is an endonuclease and a 3′ exonuclease (133). This is interesting, given that Artemis lacks a 3′ exonuclease.

PHYSIOLOGIC DNA RECOMBINATION SYSTEMS

V(D)J and class switch recombination are two physiologic breakage and rejoining systems. NHEJ carries out the rejoining phase.

V(D)J Recombination

V(D)J recombination is one of the two physiologic systems for creating intentional DSBs in somatic cells, specifically in early B or T cells for the purpose of generating antigen receptor genes. RAG1 and RAG2 (both only expressed in early B and T cells) form a complex that can bind sequence specifically at recombination signal sequences (RSSs) that consist of a heptamer and nonamer consensus sequence, separated by either a 12- or 23-bp nonconserved spacer sequence (**Supplemental Figure 3**). [HMGB1 or -2 is thought to be part of this RAG complex on the basis of in vitro studies (136).] A given recombination reaction requires two such RSS sites, one 12-RSS site and one 23-RSS site (the 12/23 rule). The RAG complex initially nicks directly adjacent to each RSS and then uses that nick as a nucleophile to attack the antiparallel strand at each of the non-RSS ends (137). The two non-RSS ends are called coding ends because these regions join to encode a new antigen receptor exon. The nucleophilic attack generates a DNA hairpin at each of the two coding ends. The NHEJ proteins take over at this point, beginning with the opening of the two hairpins by Artemis:DNA-PKcs and followed by NHEJ joining (37). Like vertebrate NHEJ, most coding ends do not share significant terminal microhomology (45, 138). The NHEJ junctions formed in V(D)J recombination have proven to be useful for understanding NHEJ more generally.

The DSBs at the two RSS ends are called signal ends, and these are blunt and 5′ phosphorylated (139, 140). In cells that express terminal transferase, nucleotide addition can occur at these ends (141). But these ends only rarely suffer nucleolytic resection, presumably because of tight binding by the RAG complex (137). Joining of the two signal ends together

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to form a signal joint is also reliant on Ku and the ligase IV complex, but joining is not dependent on Artemis or DNA-PKcs (65, 137). [The fact that DNA-PKcs is required for coding joint formation (for Artemis:DNA-PKcs opening of hairpins), but not for signal joint formation, was a point of importance in the original description of scid mice (142). Scid mice have a mutant DNA-PKcs gene (143). Artemis-null mice behave similarly (63).]

Class Switch Recombination

CSR occurs only in B cells after they have already completed V(D)J recombination. It is the second of the two physiologic forms of DSB formation in somatic cells (64). CSR is necessary for mammalian B cells to change their immunoglobulin heavy chain gene from producing Igμ for IgM to Igγ, Igα, or Igε for making IgG, IgA, or IgE, respectively (**Supplemental Figure 4**. Follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**). The process requires a B cell–specific cytidine deaminase, AID, which converts C to U within regions of ssDNA. In mammalian CSR, the single strandedness appears to be largely owing to formation of kilobase-length R loops that form at specialized CSR switch sequences because of the extremely (40% to 50%) G-rich RNA transcript that is generated at these specialized recombination zones (144, 145). This permits AID action on the nontemplate DNA strand. RNase H can resect portions of the RNA strand that pairs with the template strand, thereby exposing regions of ssDNA for AID action on that strand also. Once AID introduces C to U changes in the switch region, then UNG converts these to abasic sites, and apurinic/apyrimidinic endonuclease (APE1) can, in principle, nick at these abasic sites. Participation of other enzymes, such as Exo1, may assist in converting the nicks on the top and bottom strands into DSBs. NHEJ is largely responsible for joining these DSBs, but as mentioned above, elegant work has demonstrated the role of either ligase I or III, when ligase IV is missing (70).

CHROMOSOMAL TRANSLOCATIONS AND GENOME REARRANGEMENTS

Chromosomal translocations and genome rearrangements can occur in somatic cells, most notably in cancer. In addition, such genome rearrangements can occur in germ cells, giving rise to heritable genome rearrangements. Although the breakage mechanisms vary, the joining mechanism is usually via NHEJ.

Neoplastic Chromosomal Rearrangements

The vast majority of genome rearrangement-related DSBs (translocations and deletions) in neoplastic cells are joined by NHEJ, even though there is ample opportunity for participation of alternative ligases, if ligase IV is missing, as in experimental systems or extremely rare patients (66, 70). The breakage mechanisms in neoplastic cells include the following: random or near-random breakage mechanisms (owing to ROS, IR, or topoisomerase failures) in any cell type and V(D)J-type or CSR-type breaks in lymphoid cells (146). The lymphoid-specific breakage mechanisms can combine antigen-receptor loci with off-target loci at sequences that are similar to the RSS or CSR sequences (18, 147). In some lymphoid neoplasms, two off-target loci are recombined, and the breakage at each of the two sites can occur by any of the above mechanisms. Sequential action by AID followed by the RAG complex at CpG sites appears likely in some of the most common breakage events (called CpG-type events) in human lymphoma (146). In both CSR-type and CpG-type breaks, AID requires ssDNA to initiate C to U or meC to T changes, respectively. Departures from B-form DNA are relevant to such sites (148, 149).

Constitutional Chromosomal Rearrangements

The breakage mechanisms in germ cells are presumably primarily due to random causes

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(e.g., ROS, IR, or topoisomerase failures). Deviations from B-DNA are known to be relevant at long inverted repeats, where the most common constitutional translocations occur. The most common constitutional chromosomal rearrangement is the t(11;22) in the Emanuel syndrome (150). In this case, inverted repeats result in cruciform formation, creating a DNA structure that is vulnerable to DNA enzymes that can act on various portions of the cruciform. Once broken, the DNA ends are likely joined by NHEJ, on the basis of observed junctional sequence features.

During evolution, some of the chromosomal rearrangements that arise during speciation are almost certain to share themes with those discussed here, including breakage at sites of DNA structural variation and joining by NHEJ. Replication-based mechanisms are also likely to be very important for major genomic rearrangements (151, 152).

CHROMATIN AND NONHOMOLOGOUS DNA END JOINING

It is not yet clear how much disassembly of histone octamers must occur at a DSB for NHEJ proteins to function. In contrast to HR, where kilobases of DNA are involved and phosphorylated H2AX (γ-H2AX) alterations are important, NHEJ probably requires less than 30 bp of DNA on either side of a break.

If randomly distributed, 80% of DSBs would occur on DNA that is wrapped around histone octamers, and 20% would occur internucleosomally. For those breaks within a nucleosome, one study showed that Ku can bind, implying that the duplex DNA can separate from the surface of the nucleosome sufficiently to permit Ku to bind (153).

Several studies propose that γ-H2AX is important for NHEJ (16, 154). Much of the evidence is based on immunolocalization studies where the damage site may have contained a mixture of HR and NHEJ events within the 2000-Å confocal microscope section thickness. Differences in access within the euchromatic versus the heterochromatic regions are likely, but even early genetic insights concerning this are limited to yeast (155).

H2AX is only present, on average, in one of every ten human nucleosomes because H2A is the predominant species in histone octamers (16). Therefore, most DSBs would occur about 5 nucleosomes away (about 1 kb) from the nearest octamer containing an H2AX that is eligible for conversion to γ-H2AX via phosphorylation by ATM or DNA-PKcs at serine 139 of H2AX. Given this substantial distance from the site of the enzymatic repair, it is not clear that such H2AX phosphorylation events are critical for NHEJ.

When DNA-PKcs does phosphorylate H2AX, this increases the vulnerability of H2AX to the histone exchange factor called FACT (which consists of a heterodimer of Spt16 and SSRP1). Phosphorylated H2AX (γ-H2AX) is more easily exchanged out of the octamer, thereby leaving only a tetramer of $(H3)_2(H4)_2$ at the site, and this is more sterically flexible, thereby perhaps permitting DNA repair factors to carry out their work (156).

Poly-ADP ribose polymerase-1 (PARP-1) is able to downregulate the activity of FACT by ADP-ribosylation of the Spt16 subunit of FACT. This may be able to shift the equilibrium of γ-H2AX and H2AX in the nucleosomes. That is, PARP-1 activation at a site of damage might shift the equilibrium toward retention of γ-H2AX in the region, perhaps thereby aiding in recruitment or retention of repair proteins (156).

Hence, FACT may initially act proximally at the closest nucleosome to exchange γ-H2AX out and leave an $(H3)_2(H4)_2$ tetramer at the site of damage for purposes of flexibility of the DNA. FACT may act more regionally (distally) to favor the retention of γ-H2AX for purposes of integrating the repair process with repair protein recruitment, protein retention, and cell cycle aspects (156).

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CONCLUDING COMMENTS

Mechanistic flexibility by multifunctional enzymes and iterative processing of each DNA end are themes that apply to all NHEJ across billions of years of prokaryotic and eukaryotic evolution. Because much of this evolution was convergent, it illustrates that these themes are important for solving this particular biological problem: the joining of heterogeneous DSBs.

SUMMARY POINTS

1. NHEJ evolved to directly repair DSBs. In haploid stationary phase organisms, there is no homology donor, and HR is not an option at all. Evolutionarily, assuming that many organisms were haploid, NHEJ likely represents a very early evolutionary DNA repair strategy.
2. In eukaryotes, most DSBs outside of S/G2 of the cell cycle are joined by NHEJ. Within S/G2 phases, homologous recombination is very active because the two sister chromatids are directly adjacent.
3. Key components of vertebrate NHEJ are Ku; DNA-PKcs; Artemis; Pol X members (pol μ and λ); and the ligase complex, consisting of XLF, XRCC4, and DNA ligase IV. Polynucleotide kinase (PNK) is important in a subset of NHEJ events.
4. Predominantly convergent evolution of NHEJ in prokaryotes and eukaryotes yielded mechanisms that reflect key themes for NHEJ and the repair of DSBs. These themes are
    a. mechanistic flexibility in handling diverse DNA end configurations by the nuclease, polymerase, and ligase activities; and
    b. iterative processing of each DNA end. Each DNA end, as well as incompletely ligated junctions, can undergo multiple rounds of revision by the nuclease, polymerases, and ligase.
5. When components of NHEJ are missing (e.g., genetically mutant yeast, or mice, or extremely rare human patients), the flexible nature of NHEJ permits substitutions by other enzymes. Rather than designate such substitutions as separate pathways (e.g., alternative NHEJ, backup NHEJ, microhomology-mediated NHEJ), one can include them as part of NHEJ but designate them as such (ligase IV-independent or Ku-independent NHEJ).
6. Terminal microhomology of one to a few nucleotides that are shared between the two DNA ends improves the efficiency of joining by NHEJ in vitro and can often, but not always, bias the outcome of the joining process toward using that microhomology in vivo. However, NHEJ does not require any microhomology in vitro or in vivo.
7. Many in vivo (even most, in vertebrate cells) NHEJ junctions have no apparent microhomology. Biochemical studies indicate that joining of fully incompatible ends can occur with absolutely no microhomology via Ku plus XLF:XRCC4:DNA ligase IV. For in vivo joins, one cannot rule out occult (inapparent) microhomology use, much of which may be polymerase generated.

FUTURE ISSUES

1. Are the two DNA ends held in proximity during NHEJ or is there synapsis? In biochemical systems, XRCC4:DNA ligase IV does not appear to require any additional

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protein to help it bring two DNA ends together. This is especially clear when there are 4 bp of terminal microhomology; in which case, addition of Ku does not markedly stimulate joining. However, at 2 bp or less of terminal microhomology, Ku does improve XRCC4:DNA ligase IV ligation. This issue is relevant to whether the two DNA ends generated at a single DSB (proximal) are joined more readily than two DNA ends that arise far apart (as in a chromosomal translocation where two DSBs are involved). The issue of whether close DNA ends are joined more efficiently than ends that are far apart is a point of active study.

2. In what ways do the DNA damage response proteins mechanistically or functionally connect with the NHEJ enzymes? NHEJ at a single DSB may be so rapid and physically confined that the damage response pathways involving ATM, the RAD50:MRE11:NBS1 complex, γ-H2AX, and 53BP1 are not activated, but this is quite unclear and subject to speculation. Experimentally or with environmental extremes, a cell may be challenged with many DSBs, in which case, activation of the damage response pathways is increasingly likely. As these activate, the impact on the enzymology of NHEJ is not entirely clear.
3. Are there additional participants in NHEJ? The Werner's (WRN) 3′ exonuclease/helicase enzyme has been proposed as one candidate, but the IR-sensitivity data fail to show a large effect (157). WRN does interact with Ku and PARP-1, but it has been proposed that this may reflect a role in replication fork repair rather than NHEJ, and this seems reasonable (158). Metnase has been proposed as a possible NHEJ nuclease and helicase, but it also has decatenating activity (159, 160). Metnase is present in humans but not in apes, mice, or apparently any other vertebrates, and there is no yeast homolog. Moreover, there is no genetic knockout to demonstrate a role in NHEJ.

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