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Choosing the right path: does DNA-PK help make the decision?

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Abstract

DNA double-strand breaks are extremely harmful lesions that can lead to genomic instability and cell death if not properly repaired. There are at least three pathways that are responsible for repairing DNA double-strand breaks in mammalian cells: non-homologous end joining, homologous recombination and alternative non-homologous end joining. Here we review each of these three pathways with an emphasis on the role of the DNA-dependent protein kinase, a critical component of the non-homologous end joining pathway, in influencing which pathway is ultimately utilized for repair.

Keywords

DNA-dependent protein kinase; non-homologous end joining; DNA double-strand break repair

1. Introduction

The DNA contained within our cells is constantly exposed, and surprisingly vulnerable, to a variety of agents that can cause DNA damage. Given the critical nature of maintaining DNA, both for cell survival and for genetic fidelity, it is not surprising that organisms have evolved a variety of complex and highly regulated mechanisms that function to repair DNA damage. Among the many types of DNA lesions that can occur, the DNA double-strand break (DSB)[#] is arguably the most deleterious. It has been reported that even a single DSB can be fatal to a cell if left unrepaired [1], and misrepair of DSBs can result in large-scale chromosomal rearrangements that may ultimately lead to carcinogenesis.

DNA DSBs are generated both by exogenous as well as by physiologic sources. Examples of exogenous agents include ionizing radiation (IR), which is frequently used as a form of cancer treatment and can also be generated by cosmic rays or by decay of heavy metals; and genotoxic agents, such as topoisomerase inhibitors or DNA cross-linking agents, which are

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[#]Abbreviations: a-NHEJ, alternative non-homologous end joining; B-NHEJ, backup non-homologous end joining; c-NHEJ, classical non-homologous end joining; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-dependent kinase catalytic subunit; DSB, double strand break; HR, homologous recombination; IR, ionizing radiation; LigIV, DNA ligase IV; NHEJ, non-homologous end joining; RAG, recombination activating gene; RSS, recombination signal sequence; VDJ, variable diversity joining; XLF, XRCC4-like factor; XRCC4, X-ray cross complementing protein 4

often used for chemotherapy. Double-strand breaks are also generated by endogenous agents, such as reactive oxygen species, for example generated by cellular respiration; off-target actions of enzymes, such as inadvertent cleavage by nucleases; and from mechanical stress on chromosomes (reviewed in [2]).

Interestingly, even given the potentially hazardous consequences of DSBs, these DNA lesions are purposefully induced during select cellular processes. For example, DSB are intentionally induced during processes in which controlled genome rearrangements are required, like VDJ recombination or class switch recombination. In VDJ recombination, one of many V (variable) segments is joined to one of many D (diversity) and one of several J (joining) segments during early B-cell or T-cell development. The VDJ process requires that the sequences between the selected V, D, and J regions be excised. First, DSBs are generated in controlled manner through the action of the recombination activating gene (RAG) complex, at specific recombination signal sequences (RSS) adjacent to the V, D, and J segments. The selected V, D, or J segments are then rejoined by the non-homologous DNA end joining process of DSB repair (discussed further below). VDJ recombination is one method through which diversity can be generated in the variable domain of an antigen receptor. As a result of VDJ recombination, a small number of DNA sequences can translate into an enormous array of antigen receptors. Thus, this is one instance in which a potentially harmful DSB can be induced for beneficial recombination reactions.

There are two well-known pathways through which DSBs can be repaired, called non-homologous DNA end joining (NHEJ) and homologous recombination (HR). As is suggested by its name, NHEJ is a process whereby DNA ends are directly joined together without a specific requirement for sequence homology. In contrast, HR is a process whereby strand exchanges between similar or identical sequences can direct repair of DSBs. In addition to these two widely recognized pathways of DSB repair, a third less understood “alternative” pathway of DNA end joining is also beginning to be characterized. Here, the pathways that are required for DSB repair will be discussed, including a comprehensive review of “classical” NHEJ (c-NHEJ) and a brief review of HR and alternative NHEJ (a-NHEJ). Additionally, the role of the DNA-dependent protein kinase, DNA-PK (a critical component of the c-NHEJ pathway) in determining which of these pathways is ultimately utilized for repair will also be discussed.

2. DNA Double-Strand Break Repair Pathways

Given the potential severity of persistent unrepaired DSBs, it is perhaps not surprising that in mammalian cells, there are at least three pathways that function to repair these toxic DNA lesions (Fig. 1). What is somewhat surprising however, is how mechanistically and functionally distinct these pathways are. In general, NHEJ joining (either classical or alternative) is considered to be the most straightforward process for repairing DSBs, because at the most basic level, NHEJ entails simple ligation of two DNA ends. However, since many DSB ends contain non-ligatable groups, the situation is often complex, and requires more than just a basic end joining reaction for repair. On the other hand, the search for homology required for repair through HR is mechanistically complex. Nevertheless, during certain phases of the cell cycle, i.e. S and G₂, the newly replicated daughter strand of the sister chromatid provides an easily accessible homologous segment of DNA that can be used for homology-directed DSB repair.

NHEJ does not require sequence homology between the DNA ends as a prerequisite for ligation, (though, as discussed below, joining can be influenced by small areas of microhomology near the ends of a DSB). As a result, this process can be error-prone. Although NHEJ does have the potential to introduce harmful mutations, since much of the

genome in higher eukaryotes consists of non-essential DNA, the consequences of introducing mutations are not always detrimental. In fact, in some instances, induction of mutations through DSB repair by NHEJ can even be beneficial, for example in VDJ recombination (discussed briefly above and reviewed in [3]), where error-prone joining of V, D, and J segments adds considerable diversity to the immune repertoire. In contrast, HR is generally considered to be an error-free process. However, when a homology donor is not nearby, as is the case in G₀ and G₁, inappropriate recombination between non-homologous chromosomes or between repeated sequences on the same chromosome, can result in translocations or deletions/inversions, respectively.

Despite the fundamental differences between pathways of DSB repair, it has become clear that these pathways compete to repair DSBs. However, how a cell chooses whether to repair a DSB through NHEJ or HR, is still relatively unknown and has become an active area of investigation (see below).

3. Non-Homologous End Joining

3.1. Ku

The initiating event for NHEJ is recognition and binding of the free DNA DSB ends by a heterodimeric complex of the Ku70 and Ku86 (also called Ku80) proteins. Ku was first characterized as an autoantigen in patients with autoimmune disease, the name “Ku” is derived from the surname of the patient in which the complex was discovered [4]. Ku is an abundant protein complex in humans (~400,000 molecules per cell) that has a strong affinity for DNA ends ($\sim 10^9 \text{ M}^{-1}$) [5–7]. Based on these attributes, it is generally accepted that Ku is one of the first complexes to bind to the DNA ends generated by a DSB. The Ku heterodimer forms an asymmetric ring that can encircle double-stranded DNA ends in a sequence independent manner by making contacts with the DNA backbone (and not with the DNA bases) [8]. Ku can also bind to a variety of double-stranded end structures, including blunt ends, ends with 3' or 5' overhangs, and covalently closed hairpin ends, making Ku ideal for initiating repair of the diverse types of DSB ends induced by DNA damaging agents [7,9–11].

3.2. DNA-PK

After DNA ends have been recognized and bound by the Ku heterodimer, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) is recruited to the DSB through its interaction with Ku and DNA [DNA-PK implies a complex of Ku and DNA-PKcs]. At 465 kDa, DNA-PKcs is one of the largest kinases identified to date, and it is the only kinase that is absolutely dependent on DNA binding for activity. Based on protein sequence similarity, DNA-PKcs has been placed into the phosphatidylinositol 3-kinase-related kinase (PIKK) family (reviewed in [12]). Interestingly, the catalytic domains of the PIKKs are similar to those of the lipid phosphatidylinositol 3-kinase family, rather than to the classical eukaryotic protein kinases. However, DNA-PKcs, like other members of the PIKK family, shows no propensity for lipid phosphorylation. Instead, DNA-PK is a genuine protein kinase with a strong preference for phosphorylating serine and threonine residues that are followed by glutamine or, less commonly, a hydrophobic residue.

Whether recruitment of DNA-PKcs to Ku-bound DNA ends results as a consequence of a conformational change induced in Ku upon binding to DNA, or it is simply a reflection of a basic requirement for both DNA ends and Ku, is not completely clear. Structural studies indicate that Ku maintains its conformation in the presence and absence of DNA [8]. However, the C-terminal region of Ku86, which is important for interaction between Ku and DNA-PKcs [13,14], is absent from these structures. Nevertheless, under physiological conditions, Ku and DNA-PKcs do not form stable complexes in the absence of DNA [15]

and DNA-PKcs only binds to DNA transiently in the absence of Ku [16]. Therefore, DNA-PKcs must be recruited to the site of a DSB through interaction with Ku-bound DNA ends.

Once DNA-PKcs is recruited to the site of a DSB, Ku translocates inward, approximately one helical turn, allowing DNA-PKcs to contact an ~10 base pair region at the DNA termini [17]. Although DNA-PKcs can bind to double-stranded DNA (dsDNA) ends and be activated under low salt conditions [15,18], the presence of Ku stimulates this kinase activity significantly [15,18]. At physiological conditions therefore, it is considered that the Ku 70/86 heterodimer, together with DNA-PKcs forms the heterotrimeric DNA-PK holoenzyme. It has been shown that two molecules of DNA-PKcs bound to opposing sides of a DSB can interact, contributing to synapsis of the broken DNA ends [19]. The interaction of two molecules of DNA-PKcs stimulates the enzymatic activity of this kinase, leading Chu and colleagues to suggest that end-bridging through synapsis is required for full kinase activation. However, other studies are more consistent with DNA-PK being activated *in cis* by the DNA to which it is bound [20,21].

How DNA-PK activates and de-activates has been investigated for decades. Although it is clear that DNA binding is absolutely requisite for activation and that autophosphorylation induces dissociation of DNA-PKcs from Ku bound DNA, the precise mechanistic basis for either of these phenomena is unknown. Recent advances in structural biology of the complex show that DNA-PKcs consists of two major domains. The kinase domain is separated from the large N-terminal domain that includes two long arms into which DNA is proposed to thread [22–24]. A putative DNA binding domain exists at the back of this pocket. Interestingly, the extreme N terminus is proposed to be exposed just at the end of these ‘pincers’. Unexpected data from our laboratory suggests that a conformational change at the extreme N terminus induces kinase activation (even in the absence of DNA). Briefly, we have found that immobilizing DNA-PKcs via an N-terminal tag results in constitutive kinase activation in the absence of Ku or DNA (K.M. unpublished). These data suggest that an N-terminal conformational change (likely induced by DNA binding) activates the kinase. It follows that phosphorylations within this region might regulate kinase activation and complex stability, and indeed we have recently identified phosphorylation sites within this region that have a profound affect on kinase activation [25].

The exact role for DNA-PK in NHEJ is still not completely understood. It is clear that the enzymatic activity of DNA-PK is required for NHEJ, because eliminating the kinase activity of DNA-PK renders cells extremely sensitive to agents that induce DSBs and unable to complete VDJ recombination [26,27]. However, although a number of DNA-PK phosphorylation substrates have been identified, including proteins known to be involved in NHEJ such as Ku70 and Ku86 [28], Artemis [29,30], the X-ray cross complementing protein 4 (XRCC4) [31], the XRCC4-like factor (XLF) [32] and DNA ligase IV (LigIV) [33], phosphorylation of these sites by DNA-PK is not required for successful NHEJ [30,32–35]. In fact, the only physiologically relevant target of phosphorylation by DNA-PK to be demonstrated, to date, is autophosphorylation of the catalytic subunit itself [36–42].

Thus far, studies of DNA-PKcs have demonstrated at least three critical functions for autophosphorylation: 1) regulation of end processing, 2) enzyme inactivation, and 3) complex dissociation (summarized in Fig. 2). An important physiological consequence of DNA-PKcs autophosphorylation is regulation of DNA end access. It is critical for DNA ends to be protected from unwanted nucleolytic activity in order to prevent their degradation and preserve genetic integrity. However, at some point, the DNA ends have to be made accessible for subsequent processing and ligation reactions (see below). The autophosphorylation status of two clusters of residues, termed ABCDE and PQR, seems to be the most important requirement for regulating DNA end access (reviewed in [43]). The

ABCDE cluster consists of six sites, spanning residues 2609–2647, each of which have been shown to be autophosphorylated both *in vitro* and *in vivo* in response to DNA DSBs [36–38,42,44]. The PQR cluster consists of five sites that span residues 2023–2056 [40]. Of the five sites in the PQR cluster, only S2056 has been shown to be autophosphorylated *in vivo* in response to DNA damage [40–42,44]. However, both mutational studies and *in vitro* assays suggest that, like the six ABCDE sites, the PQR sites are redundant with one another and function as a cluster. Mutational analysis of the ABCDE and PQR clusters has demonstrated that phosphorylation within the ABCDE cluster (at even one or two of these sites) promotes access to DNA ends [39,40,42,45,46]. On the other hand, phosphorylation of the PQR cluster (on even one or two of these sites) inhibits end access [40,42]. These phosphorylation events indicate an important mechanism whereby DNA-PK can protect DNA ends and then allow end processing when appropriate. Although the resolution of the currently available crystal structure of DNA-PK does not allow specific assignment of the location of the ABCDE and PQR sites, it is intriguing to speculate that these phosphorylations somehow affect the proposed DNA binding pocket (reviewed in [22]).

It has also been clear for sometime that autophosphorylation of DNA-PKcs results in loss of kinase activity that is connected with dissociation of DNA-PKcs from Ku bound DNA [47,48]. However, the residues that must be phosphorylated to cause complete kinase dissociation have not yet been elucidated. Studies using the ABCDE phospho-ablating mutant indicate that this mutant dissociates from DNA more slowly than wild type DNA-PKcs, suggesting that phosphorylation of this cluster may contribute to dissociation of DNA-PKcs [44]. Interestingly, the dissociation defect of the ABCDE mutant can be completely alleviated by blocking phosphorylation of the PQR sites, indicating that both the ABCDE and the PQR clusters contribute to DNA end affinity [44]. However, the observed dissociation defect of the ABCDE mutant is only partial thus, although the phosphorylation status of the ABCDE and PQR clusters contribute to the affinity of DNA-PKcs for Ku-bound DNA, additional phosphorylation events (not yet identified) are required for complete kinase dissociation.

Previous studies have implicated two regions, the leucine rich region, that spans residues 1503–1602, and the extreme N-terminus in contributing to the ability of DNA-PKcs to bind to DNA [44,49]. As discussed above, new data from our laboratory provides further support for an important role of the extreme N-terminus of DNA-PKcs in kinase activation ([25] and K.M. unpublished). We have defined two additional phosphorylation sites in this region [“N” S56, S72] that may regulate enzymatic activity (Fig. 2). However, although phosphorylation of these sites alone impedes kinase activation, phosphorylation of the N sites does not induce dissociation [25]. Whether dissociation requires concurrent phosphorylations at multiple sites (already defined) or requires additional, as of yet undiscovered, autophosphorylation event(s) is an active area of research. Finally, we have also identified and characterized an phosphorylation site within the proposed activation or ‘T’ loop of the kinase domain [“T” site T3950]. Phosphorylation of this site results in kinase inactivation, but T phosphorylation does not affect complex stability [44]. Moreover, blocking phosphorylation at this site impairs radioresistance suggesting inactivation of the kinase by phosphorylation within the activation loop is functionally important in living cells. In sum, DNA-PKcs phosphorylations either at the extreme N terminus or in the C terminal activation domain inactivate the kinase without disrupting complex stability; at this point it is unclear whether these are autophosphorylations or are mediated by another protein kinase.

3.3. End processing factors

In theory, once the two DNA ends of a DSB are in close proximity to one another (as is the case in a synaptic complex), direct ligation of the two ends can occur. However, DSBs often contain damaged ends and are rarely readily ligatable. Furthermore, cleavage by the RAG

nucleases during VDJ recombination results in formation of unligatable DNA hairpin ends. As a result, DSB ends may require the activity of various processing enzymes before ligation can be successfully completed. Thus far, several enzymes have been implicated in DNA DSB end processing including Artemis, polynucleotide kinase phosphatase (PNKP), terminal deoxynucleotidyl transferase (Tdt), polymerases (pols) λ and μ , and most recently, the Ku complex.

Initial studies of Artemis indicated that this protein possesses a 5' exonuclease activity specific for single-stranded DNA (ssDNA) [50]. However, it has recently been reported that this 5' exonuclease activity can be separated from Artemis during purification and thus it seems that 5' exonuclease activity is not an intrinsic property of this protein [51]. Whether the 5' exonuclease activity first attributed to Artemis represents an unimportant contaminant, or results from a nuclease that is associated with Artemis and involved in NHEJ remains to be determined (reviewed in [52]). On the other hand, it is clear that Artemis acquires endonucleolytic activity on 5' and 3' overhangs, as well as DNA hairpins, when complexed with DNA-PKcs (that is phosphorylated in the ABCDE cluster) in the presence of ATP [30,50]. Studies indicate that Artemis is the nuclease required for opening the DNA hairpin ends generated during coding joint formation in VDJ recombination [50]. Consistently, mice lacking Artemis are defective in processing coding ends, which require hairpin opening [53]. Interestingly, Artemis knockout mice are also radiosensitive, suggesting that this nuclease has an additional role in NHEJ [54]. It is believed that the 5' and 3' endonuclease activity of Artemis may be requisite for removing ssDNA overhangs containing damaged DNA bases during end processing [55].

Polynucleotide kinase phosphatase is a bifunctional enzyme that possesses both a 5' kinase and a 3' phosphatase activity. Therefore, PNKP can mediate the removal of modified DNA termini to restore the 5'-phosphate and 3'-hydroxyl ends required for subsequent DNA synthesis or ligation reactions. Several lines of evidence have implicated PNKP as an end processing factor in NHEJ. *In vitro* studies demonstrated that human PNKP replaces phosphates at modified DSB end termini, but only in the presence of active DNA-PKcs and XRCC4 [56]. Furthermore, PNKP has been shown to interact with XRCC4, suggesting a mechanism that could couple end processing and subsequent ligation [57,58]. Finally, human cells with depleted levels of PNKP are sensitive to agents that induce DSBs, indicating a defect in DSB repair [59,60]. Importantly, human cells with reduced levels of PNKP demonstrate rates of sister chromatid exchange similar to wild type and moreover, reducing the level of PNKP in cells lacking DNA-PKcs does not further increase the radiosensitivity of such cells [60]. Thus, defects in DSB repair can be attributed to a role for PNKP in NHEJ rather than HR.

In addition to damaged bases and the presence of unligatable end groups, small gaps can also occur at or near the site of a DSB. Three enzymes from the Pol X family; Tdt, pol λ and pol μ , have been associated with gap filling in mammalian NHEJ (the reader is referred to a recent excellent review of polymerases in NHEJ [61]). Tdt is only expressed in developing lymphocytes where it can add untemplated junctional nucleotides (N segments) to coding joints during VDJ recombination. Pol λ and pol μ are ubiquitously expressed; these polymerases remain active even in the presence of broken and damaged templates and during times when the level of dNTPs is low (i.e. G₀ and G₁ when only NHEJ is actively repairing DSBs) making them ideally suited for replacing missing bases near DSB ends. Vertebrate cells that lack pol λ , pol μ , or both pols are not significantly radiosensitive, indicating that these polymerases are dispensable for completion of NHEJ [62–65]. However, evidence has suggested that inserting DNA bases opposite abasic sites, although potentially mutagenic, can prevent more severe deletions of DNA [66]. Therefore, it has

been suggested that although NHEJ polymerases are not required for completion of NHEJ they can contribute to the quality of repair by this process [61].

Recent studies by the Ramsden Laboratory have demonstrated that Ku possesses a previously unidentified 5'-deoxyribose-5-phosphate/abasic lyase (5'-dRP/AP lyase) activity that is required for efficient removal of abasic sites at or near the ends of DSBs [67]. Furthermore, Ku is at least ten-fold more active towards excising AP sites surrounding DSBs than other 5'-dRP lyases that are known to be involved in strand break repair, such as pol β and pol λ . Thus, in addition to having a role in binding DSB ends and recruiting NHEJ factors, the Ku complex also has a newly defined role in DNA end processing.

3.4. Ligase Complex

After DNA ends have been appropriately processed, they must be ligated together to complete DSB repair. A multitude of genetic studies conducted in mouse [68,69], chicken DT40 [70], and human [71–73] cells have implicated LigIV as the enzyme that is primarily responsible for sealing DNA ends to complete repair by NHEJ. Interestingly, in mice and chicken cells, inactivating LigIV leads to a significantly more severe phenotype than inactivating other components of the NHEJ machinery such as Ku or DNA-PKcs. Surprisingly, the severe phenotype imparted by inactivating LigIV can be lessened by simultaneously inactivating Ku [70,74]. Together, these data could suggest that when DSB repair by NHEJ cannot be completed, the presence of Ku also inhibits DSBs from being repaired by HR.

DNA LigIV consists of an N-terminal catalytic domain and two C-terminal BRCA1 carboxyl terminus (BRCT) domains [75]. The region separating the two BRCT domains is required for LigIV to interact with its partner protein, XRCC4 [76]. By itself, XRCC4 forms homodimers, which can associate to form homotetramers and higher order homo-oligomeric complexes [31,77]. When bound to LigIV, interaction with the linker region between the two BRCT motifs blocks the ability of XRCC4 to form tetramers, indicating that the active LigIV-XRCC4 complex is composed of LigIV and an XRCC4 homodimer [78,79]. Although XRCC4 itself does not have any known enzymatic activity, binding of XRCC4 can increase both the stability of LigIV [80] and the activity of this enzyme at the adenylation step [81,82]. Consistently, inactivating XRCC4 results in phenotypes reminiscent of those that result from inactivating LigIV [83]. Remarkably, studies conducted in the Lieber laboratory have demonstrated that the XRCC4-LigIV complex can seal one of the broken DNA strands of a DSB even if the other strand is not ligatable, suggesting that end processing and ligation can occur independently on each of the two broken DNA strands [84].

In addition to the XRCC4-LigIV complex, recent studies have indicated a role for a newly identified factor in NHEJ-dependent ligation. XLF (also called Cernunnos) was independently discovered by two groups in 2006 as an XRCC4 interacting factor that is inactivated in patients with NHEJ defects [85,86]. Although the exact role of XLF in NHEJ is not completely understood, XLF has been demonstrated to promote the re-adenylation of LigIV and to stimulate the activity of the XRCC4/LigIV complex toward mismatched and noncohesive ends, indicating a role for XLF in the ligation step of NHEJ [87–90]. Interestingly, much like XRCC4, XLF can form concentration dependent higher order complexes [90]. Based on structural data and mutational studies of the XRCC4-XLF interface, Andres et al. have suggested a model in which molecules of XLF and XRCC4 interact to form a filamentous structure of alternating XRCC4 and XLF dimers [90]. These data suggest that in addition to stimulating XRCC4/LigIV activity, XRCC4/XLF filaments may also have a role in facilitating DNA end alignment. Consistent with this model, XLF (in

the presence of XRCC4/LigIV) has been shown to stimulate alignment based gap filling by pols λ and μ [91].

Patients with defective XLF demonstrate lymphocytopenia as a result of extreme deficiencies in VDJ recombination. Interestingly, however, mice lacking XLF demonstrate only a modest deficiency in lymphocyte numbers and the lymphocytes from such mice, although IR sensitive, perform VDJ recombination at nearly wild type levels. On the other hand, embryonic fibroblasts derived from mice lacking XLF exhibit significant deficiencies in VDJ recombination [92]. Recent work from Alt and colleagues, showing that XLF is partially redundant with both ATM and H₂AX, may provide a potential explanation for the different effects of XLF deficiency in different species and cell types [93].

As described above, it is generally accepted that NHEJ proceeds through at least three distinct steps: (1) the DSB is recognized, (2) the damaged DNA ends are to be processed to remove non-ligatable groups, (3) the DNA ends are ligated together to complete repair of the DSB. Furthermore, much is known about the role of individual NHEJ factors in completing these steps. However, whether the factors required to complete each stage of NHEJ assemble at DSBs in a sequential manner or whether they assemble simultaneously forming an NHEJ supercomplex remains unclear. Many of the known NHEJ components interact with Ku, either directly or indirectly, suggesting that a supercomplex is likely. Furthermore, as described above, it has been demonstrated that the XRCC4-LigIV complex can seal one broken DNA strand of a DSB even if the other strand is not ligatable, suggesting that end processing and ligation can occur simultaneously [84]. However, if there is one supercomplex of NHEJ factors, which is maintained by Ku, the mechanism by which Ku would be removed from DNA once the DSB ends were ligated together remains unknown, although reports of targeted Ku proteolysis provide a potential mechanism to remove Ku from repaired DSBs [94]. Clearly, such important details concerning the order of assembly of NHEJ factors merits further studies.

Finally, in addition to the list of factors discussed above which all seem to have direct affects in end joining, work from numerous laboratories have documented roles in NHEJ for a growing list of accessory factors (perhaps “gluing factors”) or their activators, 53BP1 [95–97], H₂AX [98], the Mre11/Rad50/Nbs1 complex (MRN) [99], ATM [100] that likely stabilize synapsis of two broken ends to facilitate repair. It has been shown that Mre11 interacts with Ku70 [101]; an attractive model would be that both Ku and the MRN complex target the same DSB. MRN and subsequent activation of ATM and induction of the damage-induced foci could function to stabilize the NHEJ synapse promoting repair, consistent with data demonstrating a functional requirement for these factors for efficient NHEJ. If c-NHEJ fails, the MRN complex would be positioned to promote end joining by either HR or a-NHEJ – which each require MRN to facilitate initial end resection. (Fig. 1, discussed below).

4. Alternative End Joining

In addition to the relatively well-characterized classical pathway of DNA end joining, a much more poorly understood alternative end joining activity is also beginning to be defined. Early indications of an alternative end joining activity came from studies showing that cells deficient in classical NHEJ factors were still able to ligate DNA ends (generally) as efficiently as cells proficient in c-NHEJ [102,103]. However, sequence analyses showed that joints mediated by a-NHEJ tended to have somewhat larger deletions from each joined end (as compared to those mediated by c-NHEJ); moreover, a-NHEJ virtually always joined at regions of microhomology shared by the two DNA termini. Subsequently, many studies of a-NHEJ in VDJ recombination [104], class switch recombination (CSR) [105–108], and

in repair of I-Sce1-induced DSBs [109–111], have also demonstrated an almost absolute dependence of a-NHEJ on short sequence microhomologies at the two DNA termini. However, since c-NHEJ also routinely joins DNA ends by annealing at regions of short sequence homology (reviewed in [3]), one cannot ascribe all joints at regions of short sequence homology to a-NHEJ. In fact, structural characteristics of joints mediated by both pathways are largely overlapping with the exception that joints mediated by a-NHEJ virtually never join with absolutely no microhomology.

Despite the fact that a-NHEJ has primarily been characterized only in the absence of classical NHEJ, in most instances a-NHEJ is surprisingly robust. Much of our knowledge of what factors are absolutely required for c-NHEJ has come from studying VDJ recombination. Using a variety of techniques [transient episomal VDJ substrate assays (“Gellert Assay”) [112], rearrangement of integrated substrates, or rearrangement of endogenous genes in c-NHEJ defective animals], a plethora of data indicates that absence of any of the core NHEJ factors severely impairs VDJ joining (usually by several orders of magnitude) (reviewed in [113]). These results were initially difficult to understand because breaks that are not initiated by the RAG nuclease (for instance, restriction enzyme mediated breaks) are in large part joined at relatively similar rates in cells proficient or deficient in NHEJ, although modest deficiencies (less than one order of magnitude) have been documented in some cell types with some assays [110]. The paradox that cells deficient in NHEJ are completely unable to repair a RAG break while being quite proficient in rejoining most other DSBs was solved by work from Roth and colleges. The explanation for this discrepancy is that the RAG complex protects breaks from the a-NHEJ pathway. This phenomenon was revealed in large part by the discovery of a RAG mutant that is defective in protecting its ends from NHEJ [104], although other approaches have contributed to this model as well [114]. The mechanistic basis for the ability of RAGs to shepherd breaks away from the a-NHEJ pathway is not well understood. Thus, although inactivation of c-NHEJ suppresses end joining of RAG-induced DSBs by more than 1000-fold, end joining of other types of DSBs is only modestly reduced. For example, it has been demonstrated that in the absence of c-NHEJ, nearly 50% of switch region breaks are repaired, albeit at slower rates than what has typically been observed in NHEJ competent cells [107,108].

Alternative DNA end joining mediated by microhomology has been associated with oncogenic translocations in mammals. For instance, mice lacking Ku80, Artemis, XRCC4 or LigIV in the absence of p53 have been shown to develop pro-B cell lymphomas resulting from chromosomal translocations [115–118]. What is more, studies of mice lacking XRCC4 and p53, demonstrated that the formation of pro-B cell lymphomas is dependent on a functional RAG2 gene, providing convincing evidence that VDJ recombination mediated by a-NHEJ causes chromosomal translocations that lead to B cell lymphomas [119]. As described above, use of particular RAG mutants demonstrated that a-NHEJ can be quite robust, even when c-NHEJ is active [104]. Thus, it is believed that chromosomal translocations leading to pro-B cell lymphomas are suppressed by the RAG proteins, which shepherd DSBs into the c-NHEJ pathway and away from a-NHEJ. Indeed, it has been shown that linking DSBs to the RAG complex by directly fusing RAGs to the I-Sce1 endonuclease guides DSBs generated by this nuclease into c-NHEJ (and away from a-NHEJ) [114]. Together these data suggest that in the absence of c-NHEJ, repair of DSBs by a-NHEJ is responsible for generating chromosomal translocations. Consistently, recent studies by Simsek and Jasin showed XRCC4-LigIV suppresses chromosomal translocations generated by the a-NHEJ pathway [120].

Whether the alternative end joining activity demonstrated in the absence of c-NHEJ factors represents a distinct pathway or whether it is the result of c-NHEJ that uses an additional factor to substitute in the absence of a core c-NHEJ factor is still a matter for debate [121].

Recent studies of CSR in B cells deficient for both Ku70 and LigIV demonstrate substantial joining in the absence of both c-NHEJ factors [122]. Therefore, it seems likely that in the case of these cells, which lack both the DSB recognition complex and the end-sealing components of c-NHEJ, end joining events are mediated by a distinct, but possibly overlapping, DSB repair pathway. Finally, despite a clear preference for microhomology-mediated alternative end joining in a-NHEJ, not all joining events that occur in the absence of c-NHEJ factors require complementary base pairing to proceed (for example, see [104,109,110]). Such differences have led to the suggestion that a-NHEJ may actually consist of at least two separate pathways, both which occur in the absence of c-NHEJ factors, one of which is characterized by MMEJ [3,123].

Although details of the biochemical basis of a-NHEJ are still being deciphered, recent data implicate poly(ADP-ribose) polymerase (PARP) in detecting breaks, the MRN complex and CtIP in end processing steps, and XRCC1/LigIII in DNA end ligation (Fig. 1) (reviewed in [124]).

5. Homologous Recombination

The initiating step required for repair of a DSB through the HR pathway, is resection of the 5' ends. DNA end resection exposes an extended 3' single-strand overhang that is used to direct the search for a homologous strand of DNA, for strand invasion, and for initiating DNA synthesis. The MRN complex plays multiple roles in DSB repair, including contributing to the initial steps of DNA end resection (reviewed in [99]). Although the Mre11 component of the MRN complex possesses both endonuclease and exonuclease activities [125,126] that contribute to HR [127] a 5'-3' exonuclease activity, which is required for DNA end resection, has not been demonstrated. Instead, it seems likely that MRN contributes to end resection by recruiting or facilitating the activity of other HR factors (reviewed in [99]). One such factor is the CtBP-interacting protein (CtIP). Human CtIP promotes DSB end resection and is required for efficient HR. Furthermore, CtIP interacts with MRN and appears to promote HR through a common mechanism, suggesting that CtIP cooperates with MRN to mediate DNA end resection [128]. Interestingly, phosphorylated CtIP also interacts with the BRCA1 protein during the S and G₂ phases of the cell cycle [129], leading to the suggestion that BRCA1 also participates in DNA end resection [130].

Studies in *S. cerevisiae* suggest that optimal DNA end resection occurs through a two-step mechanism where initial, short-range resection is followed by a more extensive resection reaction that is carried out by more processive enzymes (reviewed in [131]). Thus, it is possible that in vertebrates end resection also proceeds through a two-step mechanism where initial end resection is mediated by MRN and CtIP and more extensive resection is carried out by processive nucleases. Exodeoxyribonuclease 1 (EXO1) is capable of resecting DNA DSB ends *in vitro*, and resection mediated by EXO1 is stimulated in the presence of the BLM helicase, consistent with the notion that extensive resection may be carried out by the concerted actions of these two enzymes in vertebrates [132].

Following end processing, three distinct stages of strand pairing and strand exchange occur, (reviewed in [133,134]): (1) presynaptic filament formation, (2) synapsis (3) postsynaptic DNA strand exchange (or branch migration). During presynaptic filament formation, Rad51 binds to the 3' ssDNA overhang generated by end resection at a ratio of one monomer of Rad51 per every three nucleotides, to form a nucleoprotein filament [135]. However, prior to Rad51 filament formation (immediately after end resection) the 3' single-strand overhang generated is first coated by the single-stranded binding protein, replication protein A (RPA). Paradoxically, binding of RPA to the single-stranded 3' DNA overhangs prevents secondary

structures, which is requisite for subsequent Rad51 filament formation. On the other hand, the presence of RPA also interferes with the ability of Rad51 to bind to the 3' overhangs, thereby simultaneously impeding Rad51 filament formation. It is thought that Rad51 overcomes RPA binding with the assistance of HR "mediator" proteins.

In vertebrates, several proteins have been shown to perform a mediator role in HR (reviewed in [136]). For example, the product of the breast cancer susceptibility gene, BRCA2 (or FANCD1), has recently been demonstrated to facilitate Rad51 filament formation through a multi-faceted reinforcement mechanism by promoting binding of Rad51 to ssDNA (and inhibiting Rad51 binding to dsDNA), by accelerating the rate at which Rad51 displaces RPA from ssDNA, and by stabilizing the Rad51 filament through inhibition of ATP hydrolysis [137]. In addition, several proteins that share sequence homology with Rad51 also play a role in the initial steps of HR [138,139]. In vertebrate cells, there are at least five Rad51 paralogs: Rad51B [140,141], Rad51C [142], Rad51D [143], XRCC2 [144,145] and XRCC3 [145]. Although the exact functions of the Rad51 paralogs in HR are not well understood, it is clear that the Rad51 paralogs can form heterodimeric and multimeric complexes that have distinct roles throughout HR. Much like BRCA2, a complex of Rad51B and Rad51C can promote binding of Rad51 to RPA-coated ssDNA [146].

After the presynaptic Rad51 filament has been formed, the synaptic phase can occur, which entails the search for DNA homology and formation of DNA joints (Reviewed in [147]). Studies of the bacterial recombinase, RecA, have indicated that a RecA filament contains two DNA binding sites, a primary site that holds the initiating 3' ssDNA overhang, and a secondary site that can accommodate an incoming segment of duplex DNA. The ability to bind two DNA molecules simultaneously facilitates the search for homology. It is thought that the search for homology mediated by the Rad51 filament proceeds through a "catch and release" mechanism similar to that utilized by RecA, where the incoming duplex DNA is transiently held within the secondary DNA binding site of the filament and, if no homology with the 3' ssDNA is found, the duplex is released and a new segment of dsDNA is sampled for homology.

Once a homologous piece of DNA is located, alignment of the ssDNA with the homologous portion of the duplex DNA molecule results in synapsis and formation of DNA joints. Binding of the invading ssDNA to the complementary strand in the duplex DNA excludes the homologous DNA strand, generating a displacement or D-loop. The efficiency of Rad51-mediated DNA joint formation can be stimulated by Rad54 *in vitro* [148,149]. It has been suggested that Rad54 can contribute to increased DNA joint efficiency through several different mechanisms that facilitate homologous DNA pairing [150]. First, the chromatin remodeling activity of Rad54 may aid in the removal of proteins from the target dsDNA. Further, Rad54 may introduce topological changes (negative supercoiling) that induce strand separation of the double-stranded homologous donor DNA. Finally, Rad54 may be involved in translocating the Rad51 filament along duplex DNA.

During the postsynaptic steps of strand pairing and exchange, branch migration and continued DNA strand displacement occur. Regions of heteroduplex DNA are extended as the Rad51 filament continues to invade the homologous dsDNA making more extensive contacts with the complementary strand, while simultaneously displacing the homologous DNA strand, expanding the D-loop. Following D-loop formation, several sub-pathways of HR can be distinguished (reviewed in [151]). In the classical DSB repair model, DNA synthesis occurs from the 3' end of the invading DNA strand, extending the D-loop. As the D-loop expands in size, the second 3' ssDNA overhang, generated by resection of the opposite end of the DSB, can anneal to the displaced DNA strand and serve as a second primer for DNA synthesis, resulting in the formation of two Holiday junctions. The Holiday

junctions can either be resolved or dissolved resulting in crossover or noncrossover products. Alternatively, if the expanding D-loop does not take hold of the second 3' overhang, the D-loop can be reversed, and the newly synthesized strand can anneal with the second resected DNA end in a process known as synthesis-dependent strand annealing (SDSA). In somatic cells, DSB repair is rarely associated with crossovers, suggesting that SDSA is the predominant HR sub-pathway in such cells.

6. Double-Strand Break Repair: Which pathway to choose?

How a cell chooses whether to repair a DNA double-strand break by classical NHEJ, alternative NHEJ, or by HR is a fundamental and largely unanswered question. Historically, the most popular model for how this choice is made is that there is a competition between the pathways. There is in fact considerable evidence for competition between HR and NHEJ, including reports showing that both pathways can co-localize and repair the same types of lesions [152–156], increased HR of substrates in cells that lack functional NHEJ and partial rescue of the severe phenotypes associated with deficiency of XRCC4 or ligase IV, by disruption of the DNA-PK complex (in this case, deletion of DNA-PK apparently makes DSBs more accessible to HR, abrogating the severe phenotype) [70,74].

Because of DNA-PK's cellular abundance, it seems that there must be more to repair pathway choice than simple competition. All three components of the DNA-PK complex are exceptionally abundant proteins; this is especially true in human cells. In contrast, expression of downstream NHEJ factors and most HR factors is more limited. It seems likely that in most cases DNA-PK will be the first repair factor to bind a DSB. Further, expression levels of the three component DNA-PK polypeptides are not reduced during S and G₂ when HR is more active [157]. Simply put, if the two pathways were in direct competition, DNA-PK would always win. Studies suggest that this is in fact true. NHEJ predominates to repair most DSBs in mammalian cells [158]. Even in cells deficient in c-NHEJ, a-NHEJ still predominates over HR. However, this does not negate the importance of HR; clearly, intact HR is required for organismal viability. Thus, it seems rational that a mechanism exists that facilitates HR, even if DNA-PK binds a break first.

6.1. Pathway Choice is affected by kinetics

Descriptively, it has been known for decades (using physical methods to enumerate broken DNA molecules) that the kinetics of end joining in cultured cells is bi-phasic [159,160]. The early rapid phase repairs most breaks, is active throughout the cell cycle, and has a half-time of ~10–20 minutes depending on the number of DSBs introduced. The slower second phase, is also active throughout the cell cycle, and has a half-time for repair of 60–90 minutes. More recent studies have been able to correlate repair pathways with these earlier kinetic studies. Mao et al. estimate that after introduction of a DSB, NHEJ activity at a given break can be completed within 30 minutes [158]. In contrast, HR activity at a given break, cannot be completed for ~7 hours. This report could not actually differentiate c-NHEJ and a-NHEJ; however, work from Han and Yu suggest that a-NHEJ has significantly slower kinetics than c-NHEJ, suggesting that both HR and a-NHEJ contribute to the slower phase of end-joining appreciated in earlier studies using physical methods to monitor end joining [108]. The slower kinetics of HR reported by Mao et al. is also consistent with kinetics of repair factor assembly at sites of laser induced damage in living cells. Whereas, Ku is targeted to sites of damage within minutes, Rad51 foci do not appear until two hours post damage [152]. In general, cells with defects in NHEJ are dramatically more radiosensitive than cells deficient in components of the HR pathway; thus, cell death in response to IR may be signaled if end joining does not occur rapidly after induction of DNA damage. In sum, at least a partial explanation for how a cell chooses how to repair its breaks is that repair is

compartmentalized such that NHEJ accesses ends first, but if NHEJ fails, other repair pathways then have access to ends.

As discussed above, an alternative model would be that both Ku and MRN localize to DSBs perhaps facilitated by an interaction between Ku70 and Mre11 [101]. MRN and subsequent activation of ATM and its downstream targets would promote NHEJ by stabilizing the NHEJ synapse, consistent with their positive effects on c-NHEJ. If rapid c-NHEJ fails, MRN would be positioned to promote HR or a-NHEJ, which both exhibit slower kinetics.

6.2. DNA-PK's enzymatic activity is required for the enzyme's ability to limit HR

What is not obvious from the model described above, is how NHEJ complexes are cleared from sites of damage if joining fails. Given the very high expression levels of Ku and DNA-PKcs and the rapid kinetics of NHEJ, how does a cell make sure that DNA-PK doesn't always win? This is of critical importance to the cell because there are some DNA ends that should be protected from NHEJ, for instance single double-strand ends that result from replication fork collapse. In fact, it has been shown that DNA-PK is targeted to and is activated by damage associated with replication stress that results in single double-strand ends [156]. Even more, elegant work from Glover and colleagues described remarkably frequent genomic deletions in human cells undergoing replication stress [161]. These deletions were likely mediated by NHEJ joining of single DNA ends from different stalled replication forks. It seems intuitive that mechanisms must be in place to protect these ends from inappropriate NHEJ (i.e. between ends generated by two collapsed replication forks) that would result in genomic deletions or rearrangements.

As noted above, the relative level of DSB induced HR is markedly increased in cells deficient in NHEJ. Recent studies in our laboratory have focused on whether the requirement for DNA-PK to limit HR is a reflection of failed NHEJ (providing more DSBs with which to initiate HR) or whether there is active regulation of NHEJ at a given break. Earlier studies from the Nickoloff laboratory suggested dominant negative effects of chemically inhibited DNA-PK ([162]). However, more recent mutational analyses from the Nickoloff laboratory [163], as well as both mutational and chemical inhibition studies from our laboratory [25], demonstrate unequivocally that DNA-PK's enzymatic activity is essential for its ability to inhibit HR. Further, DNA-PK's ability to inhibit HR is titratable and is regulated by autophosphorylation [25].

The primary physiologic effect of lacking DNA-PK's enzymatic activity is failure of NHEJ. What is unclear, is whether the requirement for DNA-PK's enzymatic activity to inhibit HR is simply a reflection of failed NHEJ, or is because of a DNA-PK phosphorylation event that is required to impose inhibition of HR. Although DNA-PK has been shown to phosphorylate many substrates both *in vitro* and *in vivo*, the only phosphorylation substrate of DNA-PK that has been demonstrated to result in a physiological alteration in NHEJ efficiency is autophosphorylation of DNA-PKcs itself (reviewed in [164]). We considered that the requirement for DNA-PK's enzymatic activity to inhibit HR might be explained by the requirement for a particular autophosphorylation event, and thus studied numerous autophosphorylation-ablating and mimicking DNA-PKcs mutants that vary in catalytic capacity, NHEJ proficiency, as well as in their ability to regulate end processing during NHEJ. These mutants were all tested for their ability to impede HR [25]. Fig. 2 summarizes how phosphorylation at each of these sites affects repair.

Although, previous work with the ABCDE and PQR mutants using a neo HR cassette suggested that phosphorylation at these two clusters reciprocally regulated HR (similar to how these clusters function to regulate end processing during NHEJ), technical difficulties with that assay bring the resulting data into question [40,165]. Moreover, more recent results

with a cyan fluorescent protein (CFP) based HR assay do not support the previous conclusion [25]. However, a limitation of the CFP-based assays is that accumulated HR is measured over many days. These data show that blocking ABCDE phosphorylation cannot block pursuant HR over a long time period probably since the ABCDE mutant is fully capable of undergoing autophosphorylation induced dissociation, albeit with slightly slower kinetics. Failure of NHEJ in cells expressing ABCDE eventually allows HR. Because NHEJ has rapid kinetics, it is still possible that ABCDE phosphorylations are relevant for early success or failure of NHEJ and then potential use of HR. Indeed, emerging data from Jeggo and colleagues demonstrate slowed kinetics of 3' end resection (as measured by Rad51 foci induced after damage) in cells expressing DNA-PKcs that cannot phosphorylate ABCDE [166]. So we conclude that phosphorylation of ABCDE and PQR clusters may reciprocally regulate end access at early points after damage, but since these sites are not required for autophosphorylation induced kinase dissociation, these impaired complexes that cannot regulate end access still eventually dissociate allowing other pathways access to damaged ends.

6.3. DNA-PKcs phosphorylations at JK, or T impede NHEJ and promote HR

As noted above, DNA-PKcs is autophosphorylated on numerous sites both *in vitro* and *in vivo*. We have taken a mutational approach (based on defined sites and site conservation) to try to define additional physiologically relevant phosphorylation sites. Using this approach we have identified a number of phosphorylations that clearly affect DNA-PK's function [25]. NHEJ can be efficiently impaired (while enhancing HR) by phosphorylation of three different sites within DNA-PKcs. We have previously shown that "T" (T3950) in the activation loop of DNA-PKcs is autophosphorylated *in vitro*, and is phosphorylated in living cells in response to DNA damage [44]. A phospho-mimic of that site inactivates the kinase, but does not induce dissociation of DNA-PKcs from Ku bound DNA. Thus, whereas the phospho-mimic (that lacks catalytic activity) does not inhibit HR at all, a phospho-blocking mutant of this site inhibits HR analogous to wild type DNA-PKcs. Recently, we have identified another potential DNA-PKcs phosphorylation site cluster that seems to also serve as a switch between NHEJ and HR ["JK", T946 and S1004] [25]. Unlike the first two clusters, phosphorylation at these sites does not appear to affect end processing. Phospho-mimicking JK does not affect DNA-PK's catalytic activity, but still impedes NHEJ while promoting HR. Thus, we suggest that DNA-PKcs phosphorylations at the T, J, and K sites, that inhibit NHEJ and promote HR, would provide an efficient mechanism to protect certain ends from the NHEJ pathway. Even more, blocking phosphorylation at J, K, and T results in moderate radiosensitivity suggesting that being able to disengage NHEJ is functionally relevant to cellular survival after ionizing radiation.

Another unanswered question is what induces and mediates J, K, and T phosphorylations? It is currently unclear whether these are autophosphorylations or whether they are mediated by another protein kinase. Although we have shown that ABCDE and PQR phosphorylation can occur in *trans*, *cis* phosphorylations at these sites may also occur. Indeed work from the Lieber and Dynan laboratories are consistent with *cis* activation and autophosphorylation of DNA-PK (although specific autophosphorylations were not studied). An attractive model would be that DNA-PK can be activated in *cis*; however, when activated as a single un-synapsed complex (as would occur at a collapsed replication fork) J, K, and T phosphorylations would occur, resulting in disengagement of NHEJ to prevent inappropriate joining. However, when DNA-PK synapses with another DNA-PK complex, ABCDE and PQR phosphorylations that regulate end access to the downstream NHEJ factors promotes rapid and appropriate end joining.

6.4. The requirement for DNA-PK's activity to inhibit HR is not just a reflection of failed NHEJ

As discussed above, our previous studies implicate the extreme N terminus of DNA-PKcs as being involved in complex stability. We have defined two highly conserved sites in this region ["N" S56 and S72]; whereas phospho-blocking mutants of these sites are fully competent in enzymatic activity and in NHEJ assays, phospho-mimicking mutants are severely impaired in catalytic activity [25]. Because of this impairment in enzymatic activity, it is not surprising that these mutants are also severely impaired in NHEJ assays. However, phospho-mimics of the N sites are also extremely proficient inhibitors of HR. Thus, in this case failed NHEJ does not result in an increase in HR. Combining the phospho-mimicking mutation with complete kinase inactivating mutations demonstrate an absolute requirement for catalytic activity to inhibit HR, suggesting that additional (as yet undefined) DNA-PK mediated phosphorylations are required to inhibit HR. Other NHEJ factors known to be phosphorylated by DNA-PK would be attractive candidates. However, we have eliminated phosphorylations of XRCC4, Ku70, Ku86, and Artemis as being important in inhibiting HR using similar HR assays [25]. Identification of DNA-PK phosphorylations that impede HR is the focus of ongoing research.

6.5. Does DNA-PK affect other repair pathways?

Recent elegant experiments from Adamo et al. and Pace et al. showed that the FA pathway protects single ends at collapsed forks from NHEJ [167,168]. These conclusions were largely based on phenotype rescue by deleting NHEJ in FA mutants. Importantly, these data further document that NHEJ targets these ends. It will be of considerable interest to determine whether DNA-PK that is impaired in disengaging itself (perhaps by blocking phosphorylation at J, K, or T) might potentiate FA phenotypes. Similarly, it may be interesting to determine whether FA factors are targets of DNA-PK that allow DNA-PK to enforce inhibition of HR.

If a-NHEJ is robust, but perhaps prone to inappropriate joining, it would seem logical that DNA-PK might regulate the choice between a-NHEJ and c-NHEJ. This of course may also be partially explained by failure of c-NHEJ, allowing a-NHEJ. Elegant work from Hendrickson and colleagues show that isogenic human cell strains lacking Ku (but not other NHEJ components) are remarkably proficient in a-NHEJ leading to the conclusion that Ku regulates the choice between c-NHEJ and a-NHEJ in human cells [169]. Although several reports suggest that DNA-PK may affect utilization of the a-NHEJ pathway [170,171], other studies do not corroborate this finding, leaving the question open for some debate [122]. We have previously reported that NHEJ defective DNA-PKcs mutants do not block episomal substrates [114]. Similarly, none of the NHEJ defective DNA-PKcs mutants discussed here significantly reduce rejoining of integrated I-SceI breaks in hamster cells (K.M. unpublished), suggesting that a-NHEJ may not be directly inhibited by DNA-PK.

Finally, it has long been known that DNA-PK can bind and be activated by single-strand breaks (SSBs) [172]. Data from several laboratories have provided more evidence for an interplay between base excision repair and DNA-PK. Interaction and phosphorylation of XRCC1 by DNA-PK has been demonstrated by Menissier-de Murcia and colleagues [173]. Moreover, this phosphorylation affects XRCC1 function in living cells. Consistent with these data, Georgakilas and colleagues have shown that DNA-PK facilitates repair of non-DSB oxidatively-induced clustered DNA lesions in human cells [174]. In sum, maintenance of genomic integrity requires interplay of multiple repair pathways.

7. Why is DNA-PK so abundant?

A puzzling issue in this field is why primate cells express such enormous levels of DNA-PK. While DNA-PK is fairly abundant in all mammalian cells, primate cells express at least fifty times more DNA-PK activity than rodent cells [175], but not more of the other downstream NHEJ factors. Moreover, the high levels of DNA-PK in human cells do not make human cells more radioresistant [162]. If DNA-PK does not afford human cells with particular resistance to DNA damage, why do human cells universally express such high levels of this huge complex?

Recently, van Gent and colleagues described the first DNA-PKcs mutation in a human patient [176]. This immunodeficient child was shown to have a hypomorphic mutation in DNA-PKcs that likely affected Artemis activation, but left enzymatic activity intact and imparted only mild radiosensitivity. Spontaneous DNA-PKcs null mutations have been described in three species (but not human) and mutations of other factors in this pathway (XLF, Artemis, and DNA ligase IV) have been described in numerous human patients. This led van Gent and colleagues to speculate (as we had previously) that DNA-PKcs or Ku mutations in humans are lethal. Why should this be? Perhaps DNA-PK plays a secondary role in human cells. Indeed, Hendrickson and colleagues have demonstrated a unique requirement for the DNA-PK complex in maintenance of telomeres. Interestingly, in the absence of Ku (and to a lesser extent DNA-PKcs) human telomeres are lost rapidly via a hyper-recombination mechanism [177].

As discussed above, we have shown that the ability of DNA-PK to inhibit HR is titratable [25]. Loss of critical base excision repair (BER) factors like XRCC1 results in early embryonic lethality because of proliferative defects associated with the inability to repair SSBs encountered during replication [178]. Cells lacking XRCC1 display exceptionally high levels of sister chromatid exchanges, which likely reflects increased HR initiated by persistent SSBs. The interdependence of BER and HR is underscored by the exquisite sensitivity of cells deficient in HR (because of BRCA mutations) to PARP inhibitors. We have studied DNA-PK dosage effects in the context of defective base excision repair, and find that over-expression of DNA-PK in XRCC1 deficient cells results in remarkable proliferative defects and increased sensitivity to DNA damaging agents (K.M. unpublished). These data may provide some explanation for more severe phenotypes observed in humans deficient in BER factors as compared to their murine counterparts.

We suggest that differing requirements for DNA-PK in different species may have several explanations. Perhaps the fact that DNA-PK deficiency can have different consequences in different species, correlates with expression levels, and also reflects a particular requirement to more carefully regulate HR, not only at telomeres, but also at other hyper-recombinogenic regions unique to primate genomes. In sum, it seems likely that DNA-PK deficiency is not compatible with human life. Understanding how DNA-PK functions to orchestrate NHEJ at a double-strand break and potentially promote or inhibit access of DNA lesions to other repair pathways should provide fundamental knowledge of how DNA-PK functions to maintain genomic stability.

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Fig. 1. Pathways for repairing DNA double-strand breaks

DSBs are repaired by one of three pathways, c-NHEJ (left panel) HR (middle panel) or a-NHEJ (right panel). c-NHEJ occurs with rapid kinetics throughout the cell cycle, and is initiated when DSBs are recognized and bound by the Ku 70/86 heterodimer. The MRN complex may co-localize to the same break (potentially through interaction with Ku70). Ku recruits DNA-PKcs forming the DNA-PK holoenzyme. Artemis, which is less abundant than DNA-PKcs, is complexed with DNA-PKcs. Upon recruitment to a DSB by Ku, the nuclease activity of Artemis is activated by *trans* autophosphorylation at the ABCDE cluster within DNA-PKcs. ABCDE phosphorylations promote end processing whereas PQR phosphorylations (also in *trans*) limit end processing. This provides tight regulation of end

access to various processing factors, including Artemis, PNKP, and X family polymerases. If c-NHEJ fails, the HR and a-NHEJ pathways can gain access to the break; this would be facilitated by the presence of the MRN complex. In this case, DNA-PK might be inactivated by phosphorylation at the J, K or T sites as well as other, not yet identified, sites. J, K and T phosphorylations have been shown to inhibit c-NHEJ and promote HR. It is not known whether J, K or T phosphorylations occur in *cis* or *trans*, but clearly DNA-PKcs autophosphorylates some sites in *cis* (K.M. unpublished data). Ligation is carried out by the XLF/XRCC4/LigIV complex. Filament formation by XRCC4 and XLF may also contribute to synapsis. Further autophosphorylation of DNA-PKcs induces kinase inactivation (N, J, K, and T sites) and finally dissociation (sites responsible for dissociation are not known, although phosphorylation of ABCDE may contribute to complex dissociation). Although assembly and action of the c-NHEJ pathway is presented sequentially, it is also possible that c-NHEJ factors are simultaneously recruited to the site of a DSB through interaction with Ku and/or DNA-PKcs. Such a supercomplex would likely require the presence of Ku to be maintained, and as a result may cause Ku to be “stuck” on the DNA following repair. Proteolysis could provide a mechanism for subsequent removal of Ku. If c-NHEJ fails or if the DSB is first recognized by PARP, MRN is recruited to the DSB. During the S and G₂ phases of the cell cycle, MRN along with CtIP and BRCA1 perform initial, short range DNA end resection which promotes HR (adapted from [179]). HR proceeds with slower kinetics than c-NHEJ. More extensive end resection is carried out by EXO1 in collaboration with the BLM helicase. The exposed 3' overhangs are then rapidly coated with RPA, which is ultimately replaced by Rad51 through the assistance of “mediator” proteins such as BRCA2 and/or the Rad51 paralogs. The Rad51 filament invades a homologous segment of DNA while simultaneously displacing the complementary strand forming a D-loop. Following D-loop formation several sub-pathways of HR can occur including formation of Holiday junctions or SDSA. Limited DNA end resection by MRN and CtIP can result in a-NHEJ (adapted from [179]), which can occur throughout the cell cycle and may involve ligation by the XRCC1/DNA ligase III complex; a-NHEJ also proceeds with slower kinetics than c-NHEJ.

How DNA-PKcs autophosphorylations affect DNA repair

Fig. 2. DNA-PK's autophosphorylation is functionally complex

DNA-PKcs is phosphorylated on numerous sites (likely more than 40) *in vitro* and *in vivo* [22]. Our laboratory has studied (by mutagenesis) ~20. The cartoon depicts only sites shown to affect repair in living cells. Phosphorylation at T in the activation loop of the kinase domain inactivates the kinase; thus blocking NHEJ and promoting HR. JK phosphorylation also impedes NHEJ while promoting HR; however, JK phosphorylation does not affect enzymatic activity. Phosphorylation of N impedes (but does not block) kinase activation, thus inhibiting NHEJ. However, (unlike JK, and T phosphorylations) N phosphorylation does not promote HR. Phosphorylation of sites within either of the two major clusters (ABCDE and PQR) enhances NHEJ by reciprocal regulation of end processing. None of the ABCDE or PQR sites (alone or in combination) alter enzymatic activity or mediate autophosphorylation induced kinase dissociation (from Ku bound DNA), although phosphorylation of ABCDE sites may contribute to kinase dissociation. Studies are ongoing to define autophosphorylations responsible for complete complex dissociation.