Chapter 27B. DNA double-strand break repair by nonhomologous end joining 220727cd3

Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 27B

DNA double-strand break repair by nonhomologous end joining.

DNA double-strand breaks (DSB) are notoriously toxic and difficult to repair. Evolution has managed to develop several DSB-repair pathways that operate in a wide range of organisms. Of the two major pathways, the previous chapter delt with repair by homologous recombination, while the current chapter will focus on repair by non-homologous end joining (NHEJ). NHEJ has the advantage of being able to work at any time during the cell cycle but at the cost of being error-prone by deleting a few base-pairs of DNA sequence.

How non-homologous end joining (NHEJ) and its components were discovered.

In 1983, P. A. Jeggo and L. M. Kemp at the National Institute for Medical Research in London, England isolated seven x-ray-sensitive mutants of a Chinese-hamster cell line (Figure 27B.1A.) (Jeggo and Kemp, 1983). Six of those mutant cell lines were also sensitive to several DNA-damaging drugs, including bleomycin. The mutations were all of the same complementation group, indicating that they were all mutations of the same gene. Jeggo and his colleagues went on to use the DNA filter elution methods (see Chapter 9) to show that the mutant cell lines were indeed defective in their ability to repair DNA double-strand breaks (DSB), but not single-strand breaks (SSB) (Kemp et al., 1984) (Figure 27B.1B). It took several more years to isolate that mutated gene, but by 1992, they had identified it as *XRCC5* (Jeggo et al., 1992), whose protein product came to be called Ku80 and was found to be an essential part of the NHEJ machinery.

But where did this 'Ku' come from? Surprisingly, it came from clinical studies totally unrelated to cancer or DNA repair. In 1981, Tsuneyo Mimori and colleagues at the Keio School of Medicine in Tokyo had discovered that some patients with connective tissue diseases related to systemic lupus erythematosus and scleroderma produced antibodies against a previously unknown antigen that they called Ku after the first two letters in the name of the first patient in whom they found the antigen (Mimori et al., 1981).

Mimori and Hardin, then at the Yale University School of Medicine in New Haven, Connecticut, went on to investigate Ku's structure and function (Mimori and Hardin, 1986; Mimori et al., 1986). They found that Ku actually consisted of two proteins that function together. They called the two proteins Ku80 and Ku70, after their approximate molecular weights, 80 and 70 kilodaltons. As the first clue to their role in non-homologous end joining (NHEJ), they discovered that the two proteins bind tightly to the ends of double-stranded DNA, such as would occur in DNA double-strand breaks (DSBs). Ku80 and Ku70 were found to bind to each other to form a kind of donut shape that assembled around the DNA helix near its broken end.

Once bound to DNA at a DSB end, Ku seemed to be able to slide along the DNA like a bead on a string, and would not come off until it reached a DSB or to some other kind of DNA terminus (Paillard and Strauss, 1991). Ku protein was found to be abundant in cell nuclei (Figure 27B.2) and was well placed there to search for and find DSBs and then to initiate their repair.

Next came a discovery by Carl W. Anderson at Brookhaven National Laboratory in Upton, New York, of a protein kinase in cells of several species that was strongly activated by double-stranded DNA (Chen et al., 2021b; Lees-Miller and Anderson, 1989). (A protein kinase is an enzyme that phosphorylates other proteins, sometimes also itself.) This DNA-dependent protein kinase (DNAPK) was found to bind Ku plus a 135 kilodalton protein that was the catalytic subunit that could phosphorylate selected serine or threonine amino acids in a variety of proteins. Its activity required both Ku and DNA (Lees-Miller et al., 1990; Peterson et al., 1995; Suwa et al., 1994).

A DNA-dependent protein kinase activity actually was discovered earlier, in 1985, as a kinase activity that was stimulated by double-stranded DNA to phosphorylate a variety of proteins (Walker et al., 1985). The discovery was accidental. Anthony Walker and his colleagues at Cambridge University, England, noted a kinase activity while studying the stimulation of protein synthesis by RNA. The kinase activity was not stimulated by RNA, but, surprisingly, by DNA that contaminated their RNA.

DNAPK was found to be a trimer consisting of a 135 kilodalton protein that was the catalytic subunit and the two subunits of Ku (Ku80 plus Ku70). The catalytic subunit (DNAPKcs) was active only when it was bound to DNA (Gottlieb and Jackson, 1993). Relevance to the repair of DNA double-strand breaks (DSB) came from evidence that Ku80 was the product of the *XRCC5* gene that had been found to function in DSB repair (Rathmell and Chu, 1994). These findings about Ku and DNAPK were intriguing, but much about their functions in DNA repair remained unknown or uncertain. It may well have been suspected

that DNAPK phosphorylates and thereby brings into play other components of the DSB repair machinery. Other early clues were that Ku binds to DNA double-strand ends and recruits DNAPKcs to those ends. Moreover, Ku stimulated DNAPKcs to phosphorylate various DNA-bound proteins, such as transcription factors (Dvir et al., 1992; Gottlieb and Jackson, 1993).

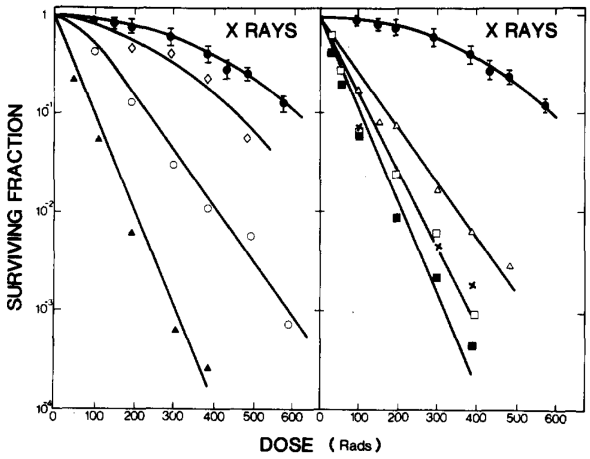

Figure 27B.1A. The increased x ray-sensitivity of mutant cells lines, relative to a non-mutated cell line (filled circles), isolated by Jeggo and Kemp in 1983 (Jeggo and Kemp, 1983). Six of the mutant cell lines were also hypersensitive to bleomycin. The mutations in the six cell lines were all of the same gene, *XRCC5*, whose protein product, Ku80. was later found to have an essential role in DNA repair by the NHEJ mechanism.

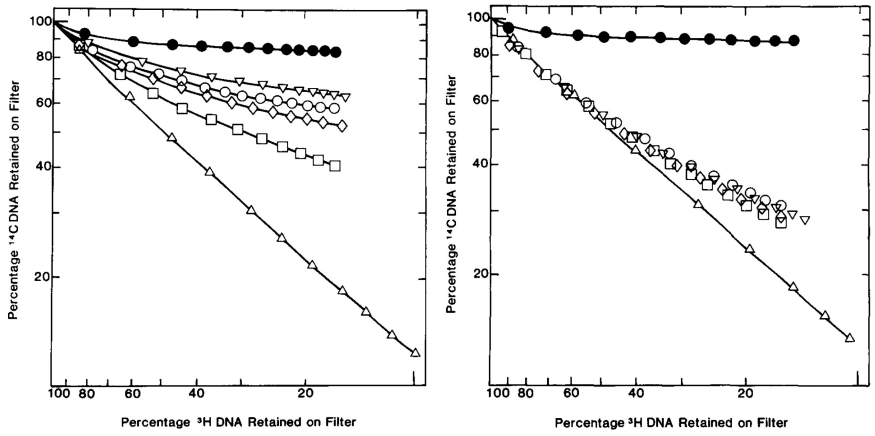

Figure 27B.1B. The panel on the right showed by neutral DNA filter elution (see Chapter 9) that an *XRCC5*-mutated cell line was defective in repair of x-ray-induced DNA double-strand breaks (DSB). (The x-ray-sensitivity of this mutant was shown by the filled squares in the *right* panel of Figure 27B.1A.) The panel on the left shows the normal repair in a non-mutated cell line. Open symbols: triangles, 0 min; squares, 20 min; diamonds, 60 min; circles, 120 min; inverted triangles, 240 min repair times after irradiation with 100 Gy of γ-rays. Filled circles, unirradiated controls. (From (Kemp et al., 1984).)

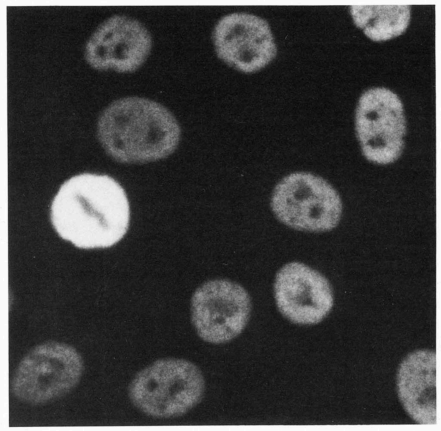

Figure 27B.2. HeLa cell nuclei were loaded with many copies of the Ku proteins, which showed up as fluorescent speckles (Mimori et al., 1986). (The cell line used was reported as Hep-2 but was later found to have been contaminated by HeLa cells – a common occurrence in those years due to the vigorous growth of HeLa cells that tended to take over cell cultures after the slightest contamination.)

How non-homologous end joining (NHEJ) repairs DNA damage.

In view of NHEJ's prominence in DNA repair – particularly the repair of the potentially disastrous DSB's – much research effort went into working out the details of that remarkable repair process.

By 1998, the binding of the Ku80-Ku70 dimer to the ends of DSBs had been firmly established (Critchlow and Jackson, 1998). Also known was that the Ku dimer can bind and activate DNAPKcs, as the catalytic subunit was called. The activation of DNAPKcs, however, also required DNA, which is why the enzyme was designated 'DNAPK' for 'DNA-dependent protein kinase.' In other words, 'DNAPK' referred to the Ku80-Ku70-DNAPKcs trimer, a catalytically active protein kinase. It was correctly supposed that the first step in DSB repair by NHEJ was the binding of the Ku80-Ku70 to the ends of the breaks and then bring in the DNAPKcs. The resulting DNA-bound trimer would then be an active protein kinase that could phosphorylate other proteins that might assemble around the DSB site in the course of the repair. The limited knowledge, as of 1998, about DSB repair by NHEJ was shown in a model by Critchlow and Jackson (Figure 27B.3.).

If we move ahead a decade to 2008, we find that researchers had come to understand the basic steps in the repair of DSBs, although the order in which some of the steps occurred was uncertain. They were amazed by the versatility of NHEJ to reconnect the ends of DSBs when the ends had a variety of abnormal structures, even when the ends seemed to be incompatible with each other – as reviewed by Michael Lieber in 2010 (Lieber, 2010). New

information had accrued by 2008 to allow Katheryn Meek and her colleagues to suggest a model of how it all happens (Figure 27B.4.) (Meek et al., 2008).

It was understood that something first had to recognize the DSB. That something was the Ku dimer consisting of two similar protein molecules, Ku80 and Ku70, which were named according to their respective approximate molecular weights, with Ku80 having some additional structure compared to Ku70. Ku was found to bind tightly to the end of a DSB. Its molecular structure had a loop into which the DNA at the end of a DSB would insert. Attached by its loop encircling the DNA, Ku could then slide along the DNA, safely attached by way of the loop around the double-helix (**B** in Figure 27B.4).

Next, enzymes to carry out the repair were inferred to assemble to form a molecular complex at the DSB. Which enzymes came into the complex would depend on the type of molecular abnormalities existing at the DSB – which is what conferred the flexibility of the NHEJ mechanism to repair a variety of problematic DSB ends. The first step in the assembly, however, was the binding of DNAPKcs to Ku and the DNA (**C** in Figure 27B.4).

The structure of Ku was revealed to have a ring that encircles the DNA near the end of the break that can glide along the DNA a short distance from the end of the DNA break; DNAPKcs bound firmly to Ku and had contact also with the DNA (**C** in Figure 27B.4). Next, the two ends of the DSB had to find and bind to each other, a process called synapsis. One way that was found to happen was through the DNAPKcs molecules at the two ends of the DSB binding to each other. According to the model, the DNAPKcs at each DNA end changes shape in a manner that allows two DNAPKcs molecules at two ends of the break to join and phosphorylate each other (**D** in Figure 27B.4).

Seven proteins were then known to take part in NHEJ: Ku70, Ku80, DNAPKcs, XRCC4, DNA ligase IV (Lig4), the nuclease Artemis, and XLF. DNAPKcs would carries out many phosphorylations to facilitate the NHEJ process. The model suggested that the two DNAPKcs molecules phosphorylate each other. The Artemis nuclease would then chew away a few nucleotides from the ends of the DNA strands until the strands from the two ends of the break found short regions of sequence homology them to join (**E-G** in Figure 27B.4). The final ligation of the DNA strand ends was found to be carried out by a complex of Lig4-XRCC4-XLF (Meek et al., 2008).

Moving ahead another decade to 2018, we find that NHEJ has much flexibility for carrying out its task under a variety of circumstances, with different sets of proteins coming in to join the ends of DNA breaks that have different molecular configurations. To begin with, there is great variability in the length of single-strand overhang (see Figure 27A.1 in Chapter 27A). Then there is the question of how far have the broken ends drifted away from each other. What happens if the ends of a break do not find each other? It was proposed that the separated ends could eventually engage in homologous recombination; however, if that occurred when the DNA had not replicated, so that there was no sister DNA strand nearby for accurate recombination, then the recombination would be apt to occur by joining with inappropriate homologous regions of the genome, thereby causing

chromosome anomalies. A particularly problematic situation would be when a replication fork encounters a DSB, or when the replication fork encounters a single-strand break (SSB) thereby generating a DSB. There appeared to be NHEJ processes configured to meet the many variation of DSB structure.

Especially noteworthy during that decade were the new molecular structures that were determined for many of the NHEJ participants. Much, although incomplete, information accrued about how those participant proteins combined in different way to carry out functions appropriate for various configurations that DSBs may have (Hnizda and Blundell, 2019; Pannunzio et al., 2018) (Figures 27B.6-7). Exactly how those proteins assembled and functioned together in the various circumstances was only partially elucidated.

As already said, the Ku70-Ku80 dimer was found to form a loop through which a DNA broken end could be threaded (Figure 27B.5). After the dimer has assembled on the DNA and moved a short distance from the end, a DNAPKcs molecule bound by associating with the Ku80 unit of the dimer. The two broken ends of the DSB, each with a Ku70-Ku80 dimer and DNAPKcs must somehow find each other and align correctly (**D** of Figure 27B.4). This process, called synapsis, was difficult to study, and some experiments even suggested that synapsis may occur by way of other proteins without implicating DNAPKcs at this step in the repair (Zhao et al., 2020). Another difficulty was that the DSB end could have many different structures, depending on what caused the break. DSB repair appeared to be able to handle different structural problems through several possible end-rejoining pathways involving different multi-protein assemblies. A common feature however was an exonuclease called Artemis that cut away abnormal DNA structures from the end-regions of the DSB. The action of Artemis was already portrayed in 2008 in the green units in **E** of Figure 27B.4 (Meek et al., 2008). By the end of 2020, many additional proteins had been found to interact with components of NHEJ, and their molecular structures were determined, but exactly how those structures assemble and function together was still not clear. It appeared that there were several ways those structures could assemble and function in NHEJ under different circumstances.

By 2022, structural studies by electron microscopy gave further detail on how DSB repair by NHEJ works, although exactly how it brings the two broken DNA ends together remained uncertain (Menolfi and Zha, 2022). At the core of the NHEJ mechanism were the Ku70-Ku80 dimer, XRCC4, Lig4, XLF, and PAXX (Figure 27B.8). Versions of these components were found in all eukaryotes (with some exceptions) (Chen et al., 2021b), pointing to an early evolution of NHEJ and the critical need of most eukaryotic organisms to be able to repair DSBs.

The picture as of 2021 was that Ku binds DNAPKcs to initiate NHEJ, and that Ku would then bind and recruit to the complex Lig4 –XRCC4 and XLF, which would bring together the DNAPKcs units from the two broken ends of the DSB and induce them to phosphorylate each other, thereby allowing them to dissociate from the complex. There were contrasting reports, however, as to whether DNAPKcs was required for DNA end-bridging during NHEJ, and exactly how these factors coordinated to bring the broken DNA ends together remained unclear (Chen et al., 2021a).

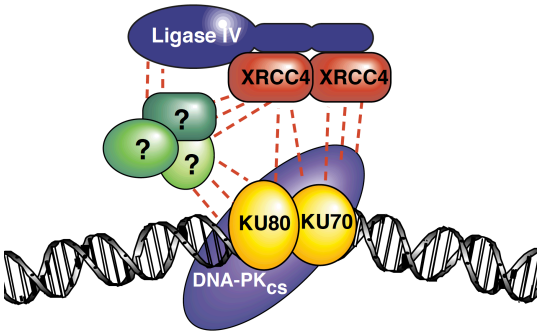

Figure 27B.3. An early model by Critchlow and Jackson in 1998 of the repair DNA double-strand breaks (DSB) by non-homologous end joining (NHEJ) (Critchlow and Jackson, 1998). They concluded correctly that the Ku80-Ku70 dimer would bind to the ends of the breaks and then recruit the catalytic subunit, DNAPKcs, to generate an active DNA-dependent protein kinase. But much about the configuration at the DSB site and how the DNA ends were brought together remained to be clarified. They also knew that DNAPKcs would recruit to the DSB site a complex consisting of Lig4 and a protein called XRCC4; they suppose correctly that the complex would come into play in final steps of the repair process to seal (ligate) the DNA strand ends to form two continuous DNA strands.

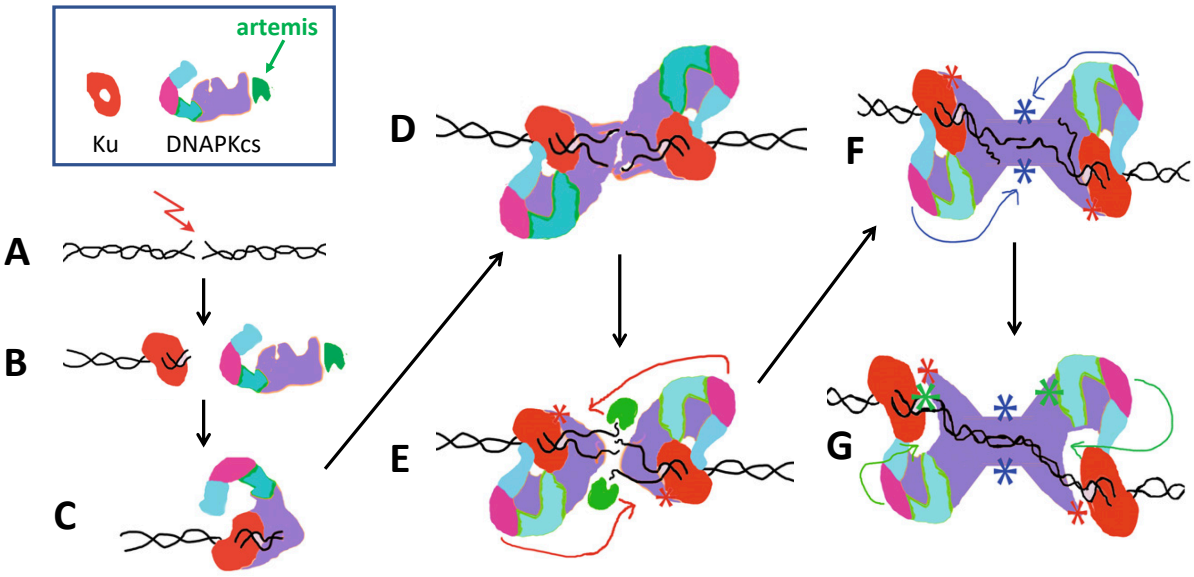

Figure 27B.4. The non-homologous end-joining (NHEJ) mechanism of DNA double-strand break (DSB) repair, as understood in 2008 by Katheryn Meek, Van Dang, and Susan Lees-Miller. (Modified and simplified from (Meek et al., 2008)). **A**, a double-strand break produced in DNA by ionizing radiation or oxidative molecules. **B**, the Ku80-Ku70 dimer bound and folded around the end of a DNA break. Ku glides a short distance (about

two turns of the DNA helix) along the DNA away from the end of the break. **C**, DNAPKcs binds to the Ku dimer at the end of the DNA break. The different colored patches represent the different functional regions (domains) of the DNAPKcs protein; the red patch is the region that has kinase activity. **D**, the two ends of the DNA break brought together by the purple domains of the two DNAPKcs molecules; the DNA helix at each end is unwound so as to facilitate the subsequent search for short complimentary sequences between the end-regions of the two ends of the break. **E**, the nuclease Artemis (green) chews away bits of nucleotide sequence from the DNA single-strand ends until a short region of sequence homology (2-4 base pairs) is found. This process is facilitated by the kinase region of each DNAPKcs molecule phosphorylating sites on the other DNAPKcs (red stars). **F**, a short region of base-pairs forms between strands from the two ends of the break. **G**, the ends of the strand breaks are sealed (ligated) by the complex of Lig4 and XRCC4 (not shown).

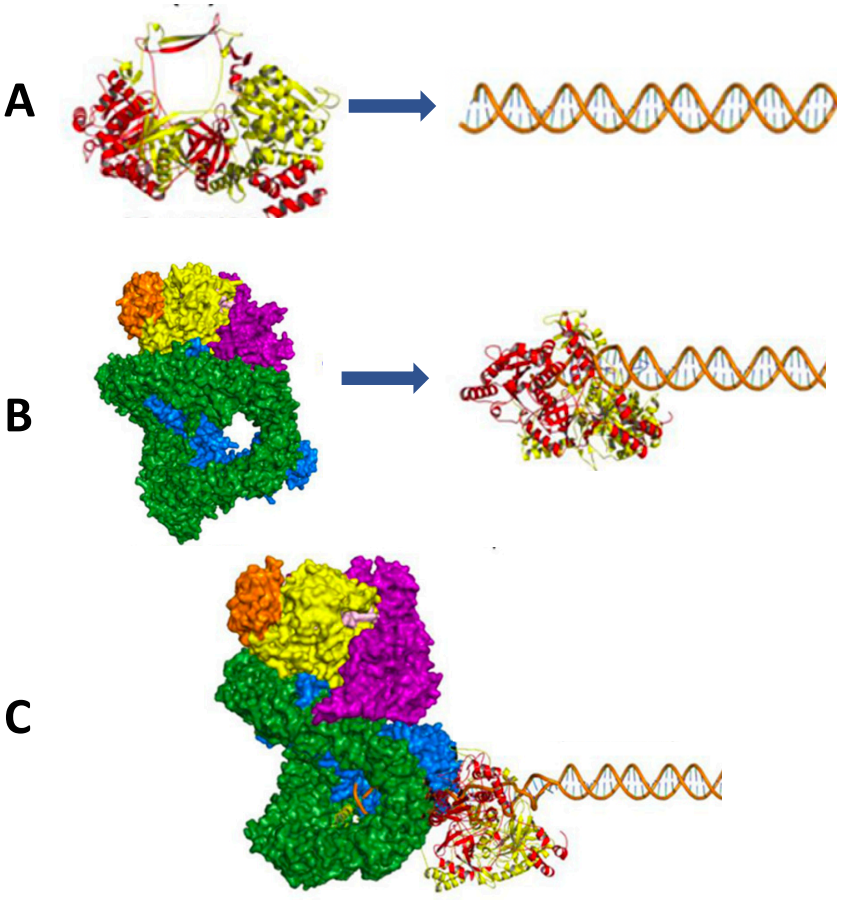

Figure 27B.5. Molecular structures of Ku and DNAPKcs showing how they assemble at the broken end of DNA, as depicted in 2018 by Nicholas Pannunzio and Go Watanabe in Michael Lieber's laboratory at the Norris Comprehensive Cancer Center in Los Angeles, California (modified and simplified from (Hnizda and Blundell, 2019; Pannunzio et al., 2018). **A**, The Ku80-Ku70 dimer binds to the broken end of a DNA double-helix. the colors red and yellow distinguish the two parts of the dimer. **B**, DNAPKcs binds to the Ku80-Ku70 dimer at the end of the DNA. **C**, the complete structure: DNAPKcs-Ku80-Ku70-DNA. The

different functional regions (domains) of DNAPKcs are shown in different colors. The Artemis nuclease (whose molecular structure had not yet been elucidated) can bind to the DNAPKcs-Ku complex at the DNA break.

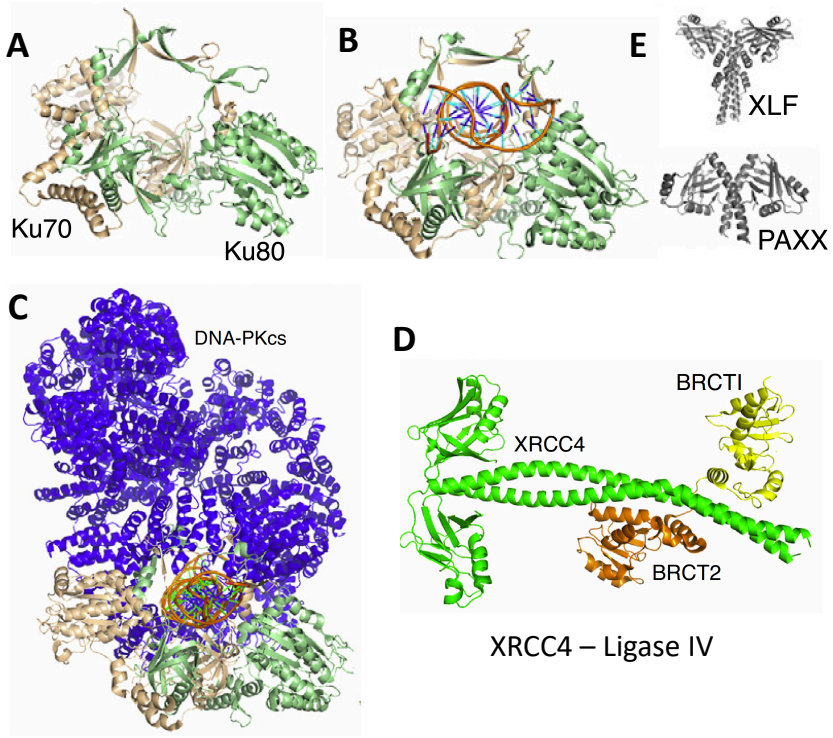

Figure 27B.6. Molecular structures of central components in non-homologous end joining (NHEJ). **A**, Ku70 and Ku80 in the structure of the Ku dimer, showing the opening through which the DNA will thread. **B**, DNA within the opening in structure **A**. **C**, DNAPKcs added to structure **B**. **D**, a XRCC4-Lig4 dimer, showing the two similar parts of the Lig4 structure (BRCT1 and 2). *Modified and simplified from (Hnizda and Blundell, 2019).*

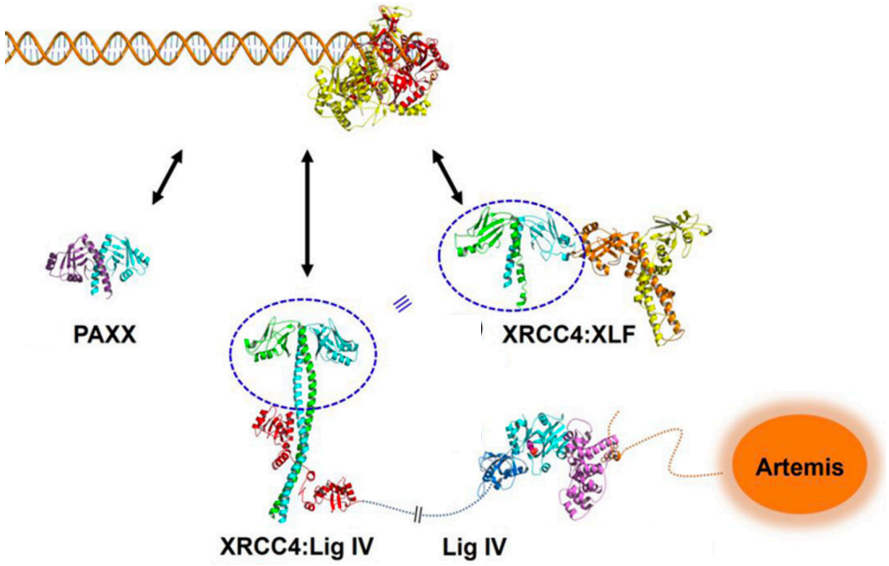

Figure 27B.7. Structures of some of the components that can assemble with Ku at the end of a DNA double-strand break (DSB). Exactly how they fit together in assemblies to handle different DSB configurations had not yet been determined. Artemis connects with Lig4 by a disordered peptide chain, but the molecular structure of Artemis was not yet known (Pannunzio et al., 2018).

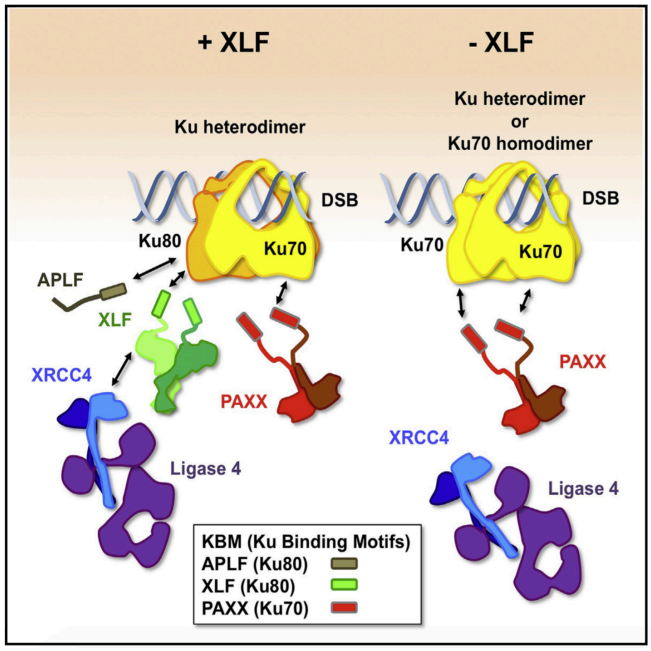

Figure 27B.8. Further details about the interactions of some of the factors implicated in NHEJ (Tadi et al., 2016). The general shape of the XRCC4-Lig4 dimer is shown in blue and purple near the bottom of the figure. The newly discovered PAXX (red) binds to the Ku70 subunit of Ku, while XLF (green) binds to the Ku80 subunit. The Ku dimer (yellow) with its loop around the DNA is nicely portrayed. In the absence of XLF, an abnormal version of Ku forms that consists of two Ku70 subunits, and PAXX can then bind to both of them (right side of the figure).

DNAPK inhibitors for cancer therapy.

Anticancer agents, such as ionizing radiation and topoisomerase inhibitors produce much of their therapeutic action by inducing DNA double strand breaks (DSB). In view of the major role of DNAPK in repairing DSBs, it was thought that DNAPK inhibitors might enhance those therapeutic actions by inhibiting DNA repair steps in NHEJ or homologous recombination, or by other actions (Mohiuddin and Kang, 2019). Supporting that idea was an early report that glioblastoma brain tumor patients treated with radiation survived longer (13 versus 9 months, p=0.02) if their tumor's DNAPK expression level was low rather than high (Kase et al., 2011). Therefore, inhibiting DNAPK expression could perhaps enhance the therapeutic action of radiation against those cancers.

As hoped, a selective inhibitor of DNAPK's kinase activity, AZD7648, inhibited DNA repair in irradiated or doxorubicin-treated cells and, in combination with a PARP inhibitor, suppressed the growth of human xenograft tumors in mice (Fok et al., 2019). By 2020, several selective inhibitors of DNAPK's kinase activity were in early clinical trial as single agent or in combination with radiotherapy, a PARP inhibitor, or a topoisomerase inhibitor (Medova et al., 2020). The antitumor actions of DNAPK inhibitor, however appeared due in part to actions on immune components in the tumor cells' environment (Nakamura et al., 2021). Structure studies of the DNAPK protein with bound inhibitor set the stage for the development of new inhibitors (Liang et al., 2022). There was a bright horizon for DNAPK inhibitors in the armamentarium for cancer therapy.

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