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The Role of Polynucleotide Kinase/Phosphatase in DNA Strand Break Repair and Its Implications in Human Diseases

Role polynukleotidové kinázy/fosfatázy při opravě zlomů DNA a její význam v lidských onemocněních

Bachelor's thesis

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Declaration of authorship

I hereby declare that I am the sole author of this bachelor's thesis. I have acknowledged all sources used and cited them correctly according to established academic citation rules. I further declare that I have not submitted this thesis, neither its substantial part to any other institution in order to obtain a degree.

Prague, 25.4. 2025 Anna Hulmáková

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Abstract

Polynucleotide kinase/phosphatase (PNKP) is a pivotal DNA repair enzyme with dual functions; DNA 3′ phosphatase and DNA 5′-kinase activity. The role of PNKP as a noncanonical end processing enzyme in DNA single-strand and double-strand break repair pathways is crucial for maintaining the stability of both mitochondrial and nuclear genomes. Mutations in the *PNKP* gene have been linked to several rare neurological disorders, including microcephaly with early onset seizures and developmental delay (MCSZ), ataxia with oculomotor apraxia 4 (AOA4), and Charcot-Marie-Tooth disease 2B2 (CMTB2B). These disorders exhibit a range of severities and pathologies from neurodevelopmental dysfunctions to progressive or mild neurodegeneration. Moreover, recent studies suggest that mutations in *PNKP* may also be linked to cancer progression. This thesis describes the role of PNKP in DNA repair pathways, its structure and enzymatic activities, and explores their connection to various pathologies, with a specific focus on the importance of phosphatase activity.

Key words: DNA repair, MCSZ, AOA4, CMTB2B, PNKP, neurological disorders

Abstrakt

Polynukleotidová kináza/fosfatáza (PNKP) je důležitý enzym přispívající k opravě poškozené DNA s dvěma funkcemi: DNA 3´ fosfatázovou a DNA 5´ kinázovou aktivitou. Role PNKP, jako enzymu zpracovávajícího nekanonická zakončení DNA při opravě jednořetězcových a dvouřetězcových zlomů DNA, je nezbytná pro udržení stability jako mitochondriálního tak jaderného genomu. Mutace v genu kódujícím PNKP jsou spojeny s několika vzácnými neurologickými onemocněními, včetně mikrocefalie s brzkým nástupem záchvatů a opožděným vývojem (MCSZ), ataxie s okulomotorickou apraxií typu 4 (AOA4) a Charcotova-Marieova-Toothova syndromu typu 2B2 (CMTB2B). Tyto onemocnění se projevují v různé míře závažnosti a patologie od dysfunkcí v neurologickém vývoji po progresivní nebo mírnou neurodegeneraci. Mimo to, nedávné studie dokonce naznačují vliv mutací v genu kódujícím PNKP na vývoj rakoviny. Tato práce popisuje roli PNKP ve drahách opravy DNA, jeho strukturu a enzymatické aktivity a zkoumá jejich souvislost s různými patologiemi, se zvláštním důrazem na význam fosfatázové aktivity.

Klíčová slova: oprava DNA, MCSZ, AOA4, CMT2B2, PNKP, neurologická onemocnění

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1 Introduction

The main function of Polynucleotide kinase/phosphatase (PNKP) involves processing noncanonical DNA break termini, which can be generated by various sources of DNA damage, such as ionising radiation, but also during abortive activity of topoisomerase I or upon endonuclease-induced DNA breakage (Inamdar *et al.*, 2002; Plo *et al.*, 2003; Wiederhold *et al.*, 2004). Utilising its unique enzymatic activities, DNA 3' phosphatase and DNA 5' kinase activities of PNKP permit the generation of suitable DNA ends for gap-filling and ligation during the DNA repair process (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). This dual functionality enables PNKP to play a pivotal role in various DNA repair pathways, including single-strand break repair, base excision repair, nonhomologous and alternative end joining (Whitehouse *et al.*, 2001; Chappell *et al.*, 2002; Wiederhold *et al.*, 2004; Audebert *et al.*, 2006). Additionally, recent data even suggest the involvement of PNKP in the maturation of Okazaki fragments, further highlighting its significance in safeguarding both the nuclear and mitochondrial genomes (Tahbaz, Subedi and Weinfeld, 2012; Tsukada *et al.*, 2025). Complete loss of PNKP has been linked to embryonic lethality in the mouse model, reflecting the essential nature in cellular maintenance and repair functions of PNKP in mammals (Shimada *et al.*, 2015).

Mutations in DNA strand break repair genes can lead to various neurological diseases. *PNKP* is one such gene known to be involved in multiple DNA strand break repair pathways, which is linked to three rare neurological disorders: microcephaly, seizures with developmental delay (MCSZ), ataxia with oculomotor apraxia 4 (AOA4) and Charcot-Marie-Tooth disease 2B2 (CMT2B2) and recent studies even suggest involvement of *PNKP* gene mutations in cancer development (Bayram *et al.*, 2022; Jiang *et al.*, 2022).

The PNKP protein contains three main domains. The kinase and phosphatase domains both have enzymatic activity, binding DNA substrates to their active sites. In contrast, the forkhead-associated domain acts as a target molecule, helping PNKP recruit to the DNA strand-break sites (Bernstein *et al.*, 2005).

Interestingly, PNKP mutations associated with AOA4, MCSZ and CMT2B2 are mainly found in the sequence encoding for the kinase domain, with only four mutations found in the sequence encoding for the phosphatase domain so far, hinting at the importance of the phosphatase activity. (Shen *et al.*, 2010; Nakashima *et al.*, 2014; Bras *et al.*, 2015; Leal *et al.*, 2018; Jiang *et al.*, 2022). The severity of a pathology not only differs between the diseases, but also between individual patients, with some even exhibiting symptoms indicative of more than one of these diseases and in some cases even having the same mutations, further complicating the understanding of PNKP's role in disease pathology (Poulton *et al.*, 2013; Taniguchi-Ikeda *et al.*, 2018; Gatti *et al.*, 2019).

This thesis aims to summarise the current understanding of PNKP´s function in DNA strand break repair and its implication in associated diseases, while also highlighting the gaps in knowledge that remain to be addressed.

2 An overview of PNKP

The human polynucleotide kinase/phosphatase (*PNKP*) gene encodes for a monomeric polypeptide with both DNA 3´phosphatase and DNA 5´kinase activity (Karimi-Busheri and Weinfeld, 1997; Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999). Human PNKP comprises three domains, two of which form the catalytic domain: the phosphatase processing 3´phosphate (3´P) termini (amino acid residues 146–337) and the kinase domain, which processes the 5´hydroxyl (5´OH) termini (341–516). The third N-terminal forkhead-associated (FHA) domain (amino acid residues 1–110) enables physical interactions between PNKP and two DNA repair scaffold proteins, XRCC1 and XRCC4, therefore permitting the recruitment of PNKP to DNA damage sites. The FHA domain is physically separated from the catalytic domain by a 35-residue cleavable linker (amino acid residues 111–145), which allows for flexibility between the FHA and the catalytic module of PNKP. The phosphatase and kinase domains are inseparable by proteolysis, suggesting these two domains are tightly bound. However, both domains still exhibit moderate rotation flexibility, possibly helping to recognise various DNA substrates (Bernstein *et al.*, 2005).

The role of PNKP in DNA repair is to transform noncanonical DNA termini generated by various damage sources into conventional DNA 3´OH and DNA 5´P termini, allowing for DNA gap-filling and ligation (Whitehouse *et al.*, 2001; Chappell *et al.*, 2002). In human cells, PNKP is predominantly located in the nucleus, which supports its role as a genomic DNA repair factor (Karimi-Busheri *et al.*, 1999). However, an additional role for PNKP in mitochondrial DNA repair has also been suggested (Tahbaz, Subedi and Weinfeld, 2012).

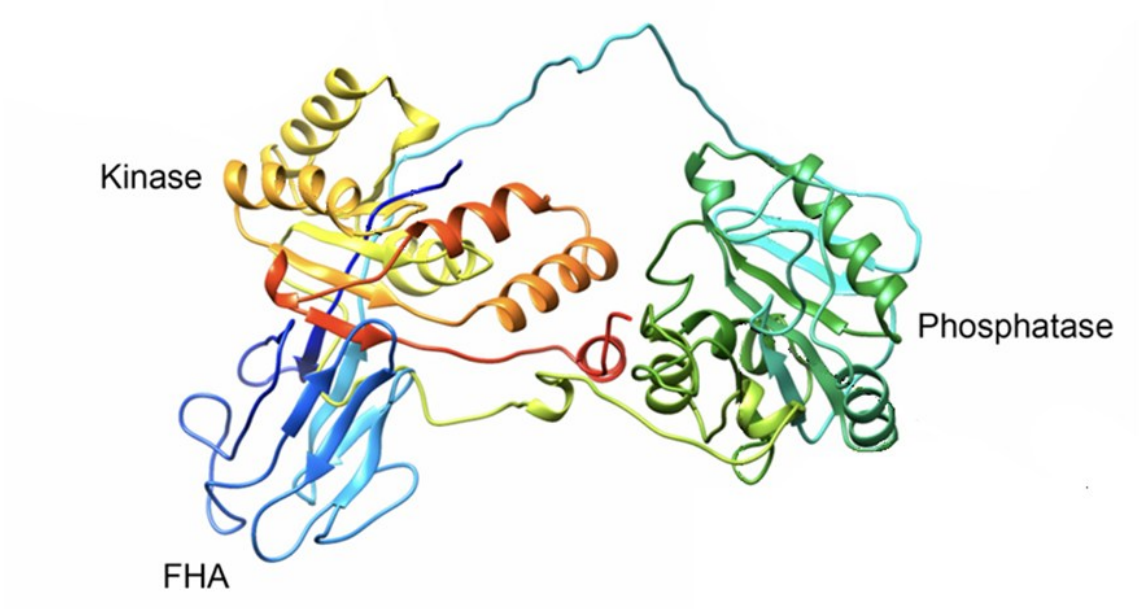

Figure 1. A 3D model visualising human PNKP. PNKP consist of three domains: the C-terminal DNA 5´kinase domain in yellow and orange, the DNA 3´phosphatase domain in green, and the N-terminal FHA domain in blue (adopted and edited from Bermúdez-Guzmán et al., 2020).

Even though PNKP is now studied mainly in the context of human genetic disorders, PNKP DNA sequence conservation is not restricted only to mammals. Homologs of PNKP have been highly conserved during evolution across various kingdoms, including Animalia, Plantae, Fungi, and Protista. Interestingly, not all PNKP orthologs have both the kinase and phosphatase domains (Jilani *et al.*, 1999; Karimi-Busheri *et al.*, 1999; Petrucco *et al.*, 2002). On the one hand, Pnkp1, the ortholog of human PNKP in fission yeast (*Schizosaccharomyces pombe)*, has both 3´kinase and 5´phosphatase domains, and even though their respective sequences differ from their human counterparts, the central binding motifs remain the same (Meijer *et al.*, 2002). On the other hand, the PNKP ortholog from budding yeast (*Saccharomyces cerevisiae)* Tpp1, contains only the DNA 3´phosphatase domain, which is also observed in plants such as *Arabidopsis thaliana* (Vance and Wilson, 2001). The reduced conservation of the kinase domain relative to the phosphatase domain could suggest a greater functional importance for DNA 3´phosphatase activity, corresponding with some of the additional data discussed below.

3 Three-dimensional structure and biochemical features of PNKP

3.1 The phosphatase domain

3.1.1 Substrate preferences

Unlike the PNKP kinase domain, the phosphatase domain does not exhibit strict substrate preferences. It efficiently dephosphorylates various substrates, including nicked or gapped double-stranded DNA substrates and single-stranded substrates of diverse lengths. The shortest substrate required for efficient phosphorylation is a three-nucleotide-long single-stranded DNA (Bernstein *et al.*, 2005).

3.1.2 Structure and substrate recognition

The phosphatase domain of PNKP belongs to the Haloacid dehydrogenase (HAD) superfamily. Even though enzymes in this family have highly variable sequences, there are several conserved residues, such as those involved in catalysis, binding to the Mg^{2+} or the substrate and intermediate, suggesting a similar reaction mechanism. Using purified muse PNKP, it has been shown that after the DNA substrate binds to the active site, there is a nucleophilic attack during which the phosphate in the DNA 3´P substrate gets transferred to Oδ1, which is one of the two oxgen atoms in the carboxylate group of aspartate (Asp) 170 side chain, creating a phosphor-aspartate intermediate in the presence of Mg2+ cofactor. Lastly, the enzyme is regenerated in a dephosphorylation reaction in which a water molecule, activated by Asp 172, attacks the phosphorus atom (Bernstein et al., 2005).

The active site of the phosphatase domain lies in a narrow, open-ended channel, in which the Mg^{2+} ion, an essential cofactor for dephosphorylation, is located. The three nucleotides on the 3´termini are bound in the channel, corresponding with the requirement for at least a 3-nucleotide-long substrate to maintain efficient dephosphorylation. However, the channel can also bind non-terminal segments of single-

stranded DNA, allowing for localisation of the 3´P termini on 3´ overhangs. Since the channel is so narrow, it fits only single-strand DNA, which is not a problem in 3´ overhangs (Garces, Pearl and Oliver, 2011), generated for example as part of the homologous recombination pathway (Zhu *et al.*, 2008), but in gapped or nicked substrates, double-stranded DNA has to be disrupted (Garces, Pearl and Oliver, 2011). Double-stranded, blunt-ended substrates have the terminal bases destabilised by PNKP. To access the narrow active site, the phenylalanine (Phe) wedge destabilises intrahelical base stacking in the substrate. The Phe305 deeper in the active site stabilises the freed single-strand substrate through stacking interaction with the base on the 3´ ends. A charged region on the other side of the cleft then interacts with the second strand, helping with the duplex DNA binding (Coquelle *et al.*, 2011). These interactions compensate for the energy needed for the base pair disruption (Havali-Shahriari, Weinfeld and Glover, 2017).

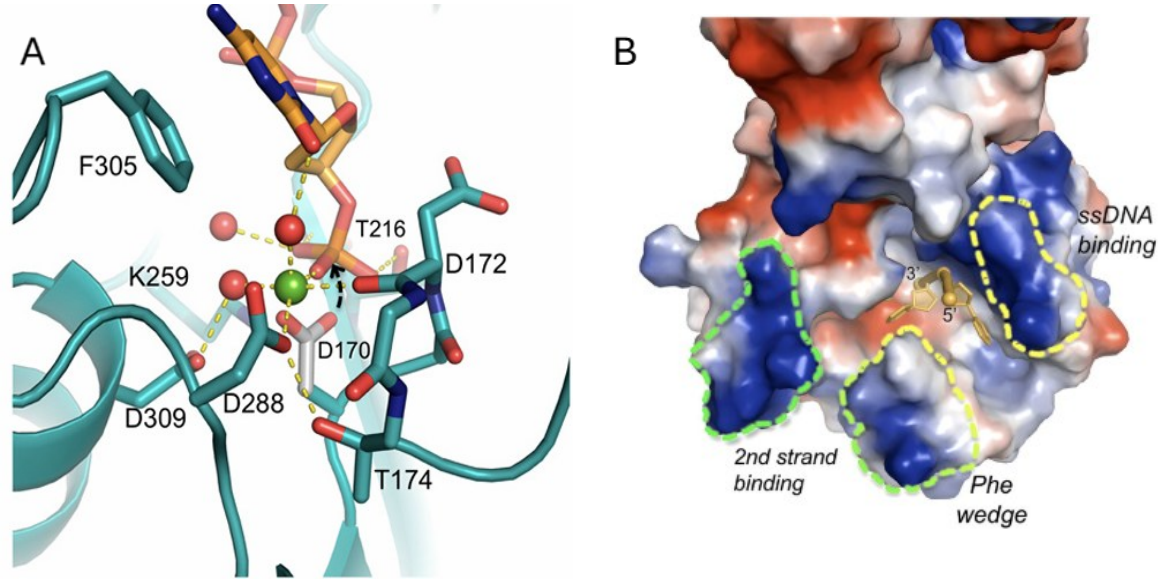

Figure 2. Structure of the phosphatase domain active site. A – A view into the phosphatase active site with bound DNA substrate. The phosphatase domain is teal, the DNA substrate orange, with the terminal 3´ phosphate having a slightly darker tone, and the green sphere is a Mg^{2+} cofactor, coordinated by the surrounding oxygen atoms, which are red. The grey amino acid side chain is the carboxylate group of Asp 170, which initiates the nucleophilic attack indicated with an arrow (adopted from Coquelle et al., 2011). B – An electrostatic surface representation of the phosphatase domain active site. The blue represents the positively charged surfaces. The phosphatase active site lies in a narrow channel, where only single-strand DNA fits. The Phe wedge disrupts the double-stranded DNA, for the 3´P to get into the active site, the disruption is stabilised by the interaction of the single-stranded DNA with the positively charged surfaces (adapted from Coquelle et al., 2011).

3.2 The kinase domain

3.2.1 Substrate preferences

The minimal length of a kinase domain substrate is eight nucleotides, as PNKP preferentially phosphorylates in the context of nicks and small gaps compared to overhanging single-stranded 5′termini and blunt-ended double-stranded termini (Karimi-Busheri and Weinfeld, 1997). Double-stranded substrates with 3′ single-strand overhang are phosphorylated as efficiently as gapped or nicked substrates, implying that 5′recessed hydroxyl termini are ideal for the kinase domain, with optimal 3′overhang being between 3 and 5 nucleotides, with 3 being the minimum nucleotide length (Bernstein et al., 2005)

3.2.2 Structure and substrate recognition

The PNKP active site is located in a long cleft with ATP and substrate binding sites on the opposite ends (Bernstein *et al.*, 2005). In the active site, two positively charged surfaces enclose the catalytic Asp396, which activates the substrate 5′ hydroxyl termini for phosphorylation. Asp396 and the two positively charged surfaces bind the substrate, the first surface interacting with the upstream portion of the substrate and the second one with the downstream portion of the 5′hydroxyl termini. The first surface interacts with the phosphodiester backbone of nucleotides near the 5′termini and the more distant double-stranded part of the substrate (6-8 nucleotides from the 5′end), explaining the minimal substrate length requirement (Bernstein *et al.*, 2009). The carboxylate group in Asp396 is oriented toward the 5′ hydroxyl termini, acting as the general base in the phosphoryl transfer reaction, taking a proton from the 5′ OH hydroxyl termini. (Garces, Pearl and Oliver, 2011). The ATP binding site is made of conserved Walker A and B motifs found in P-loop kinases, which PNKP is a part of (Leipe, Koonin and Aravind, 2003). The Walker A motif binds β and γ phosphates of ATP, and Walker motif B coordinates the Mg^{2+} cofactor, which is required for the kinase activity. Over the Walker A motif folds a lid domain composed of two alpha helices (12 and 13) (Bernstein *et al.*, 2005). The helix α13 disrupts base stacking on either strand across the site of the nick, making the DNA backbone twist around the helix surface, which exposes and positions the 5′ hydroxyl termini in the kinase active site (Garces, Pearl and Oliver, 2011).

Spectroscopic studies have shown that when PNKP binds to ATP, it undergoes conformational changes and assumes a more relaxed structure (Mani *et al.*, 2001). Several changes occurred in the kinase domain structure upon DNA and ADP binding. Catalytic Asp396 is moved to lie above the DNA terminus 5′ oxygen, and there are rearrangements for ATP/ADP accommodation and interaction (Garces, Pearl and Oliver, 2011). Another way to induce structural changes in the kinase domain is by binding the unphosphorylated XRCC1, a scaffold protein involved in DNA single-strand break repair (SSBR). While phosphorylated XRCC1 binds to the FHA PNKP domain, unphosphorylated XRCC1 binds with lower affinity to the catalytic domain of PNKP. This binding results in the enhancement of the enzymatic

activities of PNKP, even though to a lesser extent than the binding of phosphorylated XRCC1 (Lu *et al.*, 2010). The lower-affinity binding site on XRCC1 consists of three invariant phenylalanine residues(Phe172, Phe192, Phe193), two of which are part of the Rev-1 interacting RIR motif (Breslin *et al.*, 2017). However, the whole region of residues 166-436, including the BRCT1 domain, contributes to the binding (Mani *et al.*, 2019). The binding of the unphosphorylated XRCC1 also results in a change of secondary structure, specifically a decrease in the α-helical structure. (Lu *et al.*, 2010). Similarly, the α-helical structure is also decreased when ATP is bound (Mani *et al.*, 2001). These conformational changes upon binding imply that PNKP has an active and inactive conformation, similar to those of allosterically mediated protein kinases (Johnson, Noble and Owen, 1996).

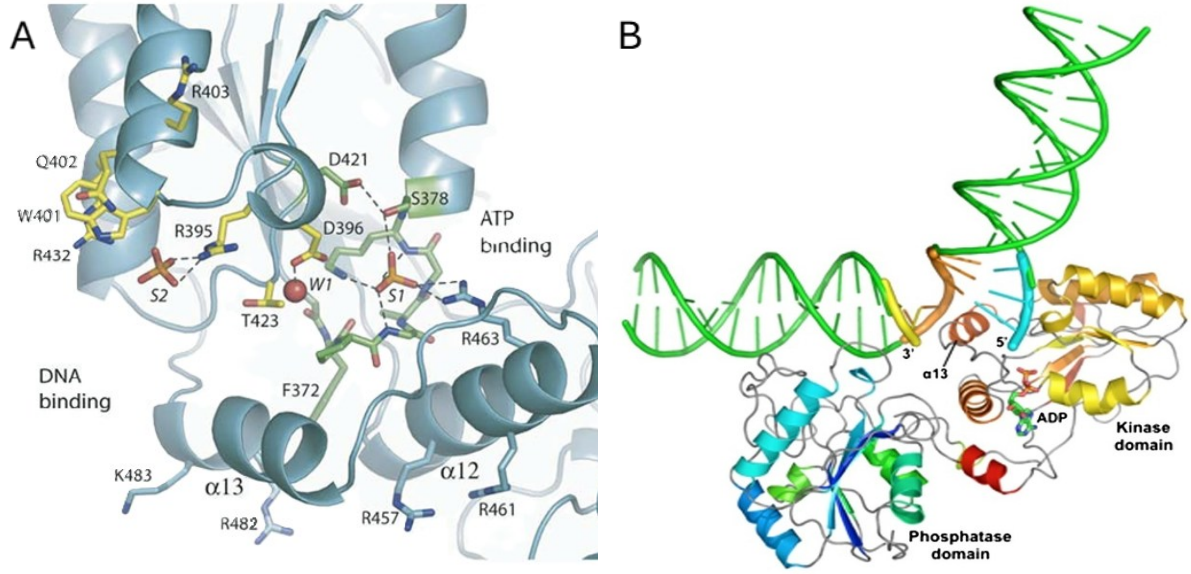

Figure 3. The structure and substrate binding of the kinase domain active site. A – A view into the kinase domain active site. The kinase domain is blue, with the interacting amino acids in yellow and the ATP binding Walter A motif in green. The orange S1 sulphur represents the β phosphate in ATP. The red sphere is a water molecule representing the DNA 5′OH termini interacting with the catalytic Asp396. The S2 sulphur atom represents the DNA sugar-phosphate backbone, being additionally stabilised by more amino acids (adopted and edited from Bernstein et al., 2005). B – Proposed model for binding a nicked DNA substrate to the kinase domain active site. To properly expose binding of the DNA 5′OH termini visualised in light blue, the helix α13, in orange, disrupts the base stacking, making the DNA backbone, also visualised in orange, twist around it (adopted from Garces, Pearl and Oliver, 2011).

3.3 Interactions between the phosphatase and kinase domain

When nicked DNA interacts with the kinase domain, helix α13 stabilises the DNA distortion and disruption of base stacking. The phosphatase backbone of the continuous strand folds over the helix, causing a ~70-degree bend. The double-stranded DNA complex downstream of the nick then interacts with the basic surface of the phosphatase domain, implying its role in supporting the kinase domain in processing nicked and gapped substrates. Structural data also suggests that for gapped and nicked substrates with 3′P and 5′OH termini, having the helix α13 blocking base stacking across the nick and

changing the direction of the substrate might enable the two active sites to interact with the termini on the same substrate at once (Garces, Pearl and Oliver, 2011).

To bind the 3′P of dsDNA substrates, PNKP might first non-specifically interact with the DNA by positively charged surfaces on both kinase and phosphatase domains in search of a strand break. When a break is detected, its flexibility allows better examination of the break and then the base pair distortion (Coquelle *et al.*, 2011; Havali-Shahriari, Weinfeld and Glover, 2017). After nicked or gapped substrates are bound, they sterically hinder the binding of a second substrate to the kinase domain. (Coquelle *et al.*, 2011). This is in agreement with the fact that when the phosphatase domain is mutated, the kinase domain activity is also negatively affected if 3′P termini are present. This is because when both 3′ and 5′ termini are present, the phosphate domain preferentially binds the substrate, sterically blocking binding to the kinase domain. Not only does the phosphatase domain activity take precedence, but it is also faster, making the kinase activity the rate-limiting step in processing these types of breaks (Dobson and Allinson, 2006). This precedence might be due to the higher frequency of generation of 3′P upon DNA damage (Lennartz *et al.*, 1975), the fact that unprocessed 3′P termini not only block ligation but also replication or the increased cytotoxicity when processing of 3′P termini on DSBs is delayed (Chalasani *et al.*, 2018).

4 The role of PNKP in DNA strand break repair pathways

Various sources of DNA damage generate noncanonical 5′OH and 3′ P DNA termini. PNKP is involved in the repair of both single-strand and double-strand breaks. Both repair pathways require downstream DNA processing mediated by DNA polymerases and DNA ligases, which are dependent on the presence of the canonical 3′-hydroxyl and 5'-phosphate DNA termini for their function (Whitehouse *et al.*, 2001; Chappell *et al.*, 2002).

4.1 Causes of DNA strand breaks

4.1.1 Oxidative DNA damage

Oxidative damage can be caused endogenously by aerobic metabolism, in which free radicals are produced (Fridovich, Irwin, 1983). In addition, ionising radiation (IR) can also cause the production of hydroxyl radicals, formed upon the radiolysis of water, which are then responsible for phosphodiester backbone breaks (Ward, 1985). SSBs caused by IR are usually repaired in time and, therefore, are not lethal for the cell (Ward JF, Blakely WF, Joner EI., 1985). However, whenever SSBR is defective, such as during PNKP downregulation, cells are more sensitive to IR (Rasouli-Nia, Karimi-Busheri and Weinfeld, 2004). DNA lesions resulting from IR exposure can have noncanonical 3 ′ and 5′ termini, which require additional processing for successful DNA ligation. Two main types of 3′ termini have been observed upon IR-induced breaks, 3′phosphate and 3′phosphoglycolate (Henner *et al.*, 1983; Buchko

and Weinfeld, 1993). While 3´P termini formation is oxygen-independent, 3´phosphoglycolate termini (3´PG) are formed solely in the presence of oxygen (Buchko and Weinfeld, 1993). In addition, 5´OH termini are present at about 10-14% of IR-induced breaks, indicating that the DNA termini are mostly 5´P (Lennartz *et al.*, 1975). IR not only induces SSBs but also clustered DNA damage, i.e. two or more closely spaced damage sites on opposing strands. These damage sites include abasic sites and oxidised bases, 20% of which become DSB when two free radical attacks on opposite DNA strands (Sutherland *et al.*, 2000). Furthermore, the radiometric drug bleomycin enables site-specific free radical attack on both DNA strands, also causing DSBs. Interestingly, bleomycin-induced DSBs are characterised by two opposite breaks, which are identical or have a 1-base 5´ stagger; while both strands have 3´ phosphoglycolate termini (Povirk, 1996).

Noncanonical DNA in the 3´and 5´ terminus must be processed for potential gap-filling and ligation. PNKP is responsible for processing 3´P termini but shows no detectable enzymatic activity on 3´PG (Inamdar *et al.*, 2002). SSBs with 3´PG termini can be processed by the enzymatic activity of APE-1 (human apurinic/apyrimidinic endonuclease 1); the enzymatic activity of APE-1 cleaves the phosphoglycolic acid and leaves 3´OH termini (Winters *et al.*, 1994). Interestingly, at DSBs, APE-1 can process PG, although with lower efficiency (Suh, Wilson and Povirk, 1997). In this case, the repair of 3´ overhang termini is partially facilitated by human tyrosyl-DNA phosphodiesterase (TDP1), which catalyses the removal of glycolate, forming 3´P termini susceptible to PNKP activity (Inamdar *et al.*, 2002). Interestingly, cells derived from patients suffering from spinocerebral ataxia with axonal neuropathy 1 (SCAN1) carrying mutations in TDP1 are incapable of 3´PG processing, showing a visible impairment; however, they are surprisingly just slightly more radiosensitive. These data suggest the existence of an alternative pathway for overhang 3´PG termini processing (Zhou *et al.*, 2005). That pathway might be provided by Artemis nuclease. Artemis can efficiently process overhang termini at least 3 nucleotides, and even shorter and blunt ends can be processed, although at a much slower rate (Povirk *et al.*, 2007).

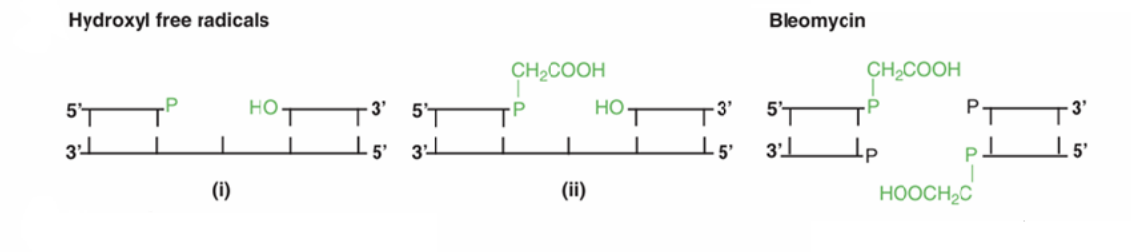

Figure 4.Types of termini generated by oxidative damage. Free radicals generate two types of termini:(i) 3´phosphate and (ii) 3´phosphoglycolate. Radiometric drug bleomycin induces a specific free radical attack on both strands, generating two 3´phosphoglycolate termini on opposite strands (adapted and edited from Weinfeld et al., 2011)

4.1.2 Abortive topoisomerase I and topoisomerase II activity

The main function of the enzyme topoisomerase I (TOP1) is to relieve the DNA supercoiling ahead of replication and transcription complexes. Human TOP1 binds the double-stranded DNA and cleaves one of the duplex strands, forming a covalent phosphodiester bond by transesterification between the hydroxyl group of catalytic tyrosine T723 and the 3´phosphate of the residue on the site of the break, forming a cleavage complex. After the release of the topological stress, the strands are ligated, and TOP1 is released (Stewart *et al.*, 1998).

Under normal conditions, TOP1 cleavage complexes (TOP1cc) are transient and almost undetectable. However, when there are topoisomerase inhibitors such as camptotecin (Hsiang *et al.*, 1985) or structural damage such as base pair mismatch or pyrimidine dimers (Yeh *et al.*, 1994; Lanza *et al.*, 1996), the final ligation step can be slowed down or blocked. The arrest of the final ligation step can lead to an abnormally high number of TOP1cc that can be detected as protein-linked SSBs. These unresolved SSBs result in a stable cleavage complex that, upon collision with a DNA polymerase, can cause replication fork stalling and subsequent collapse, which can then result in conversion of the SSBs to DSBs (Covey *et al.*, 1989). Replication fork stalling, however, is not caused only by TOP1cc but can also happen, for example, as a result of DNA lesions, collision with transcriptional machinery or microsatellites, all of which can lead to DSBs, if the stalled fork is not stabilised or restarted (Zeman and Cimprich, 2014; Gadgil *et al.*, 2020). Overall, if not repaired, the TOP1cc can result in chromosome damage and even cell death (Froelich-Ammon and Osheroff, 1995).

To resolve TOP1cc and prevent ensuing genomic instability, it is essential for the cell to enzymatically process stable TOP1cc. The TOP1 protein in TOP1cc is mostly degraded by the proteasome, leaving a short peptide which is then further processed by TDP1. TDP1 cleaves the tyrosine-DNA phosphodiester bond between DNA and the peptide, leaving a 3´P terminus on the DNA (Yang *et al.*, 1996). Consequently, the new 3´P termini can be processed by PNKP, which is compatible with DNA ligation and completion of DNA repair (Plo *et al.*, 2003).

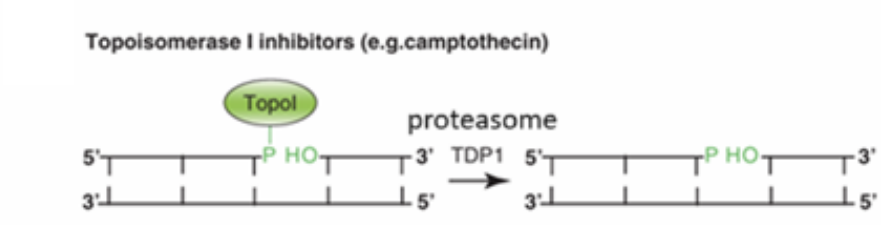

Figure 5. Types of termini generated by TOP1 abortive activity. Topoisomerase I inhibitors or DNA disruptions can lead to stable TOP1cc, which is further degraded by the proteasome, leaving a peptide residue processed by TDP1, generating 3´phosphate termini (adopted and edited from Weinfeld et al., 2011)

Topoisomerase II (TOP2) catalyses topological changes in DNA, which are essential for processes such as replication and chromosome segregation. Unlike TOP1, TOP2 forms transient double-strand breaks (DSBs) as part of its catalytic activity. However, if the TOP2 DNA cleavage complex (TOP2cc) becomes stabilised, for example, by a cancer treatment drug, etoposide, DNA re-ligation is prevented, and the DSBs can become persistent and cytotoxic (Wu *et al.*, 2011; Gómez-Herreros, 2019). The stable TOP2cc is removed by the proteasome, leaving a covalently bound peptide residue on the 5´termini (Alchanati *et al.*, 2009). The residual peptide is then either cleaved by MRE11 or tyrosyl-DNA-phosphodiesterase 2 (TDP2), leading to subsequent DSBR by NHEJ (Ledesma *et al.*, 2009; Hoa *et al.*, 2016).

4.1.3 Endonuclease-induced DNA breaks

Bifunctional DNA glycosylases/AP lyases are essential DNA repair enzymes in BER. There are two classes of DNA glycosylases/AP lyases that differ in the mechanism of enzymatic reaction and the type of termini generated. The first class of glycosylases belongs to the same group as the *E.coli* Nth glycosylase, which uses β-elimination to cleave the DNA strand at the AP site, generating 3´ phospho α,β-unsaturated aldehyde termini (Nash *et al.*, 1996). The second class of glycosylases belongs to the group of *E. coli* Fpg and Nei, which use βδ elimination for the DNA cleavage and generate 3´P termini (Zharkov and Grollman, 2005). Interestingly, in *E.coli* the Exonuclease III Xth can efficiently process both types of termini and generate 3´OH termini (Demple and Harrison, 1994); however, in mammals, APE1 can process termini generated by β-elimination but has only a weak 3' phosphatase activity (Wiederhold *et al.*, 2004). The human NTH1 and OGG1 belong to the Nth superfamily (Nash et al., 1996); therefore, after DNA cleavage, the 3' termini are efficiently processed by APE-1 (Wiederhold et al., 2004). In contrast, APE-1 processing of 3' P termini generated by mammalian glycosylases is undetectable (Wiederhold *et al.*, 2004). Therefore, PNKP is required to process the termini generated by NEIL-1 or NEIL-2 as part of the APE-independent BER pathway(Wiederhold *et al.*, 2004; Das *et al.*, 2006).

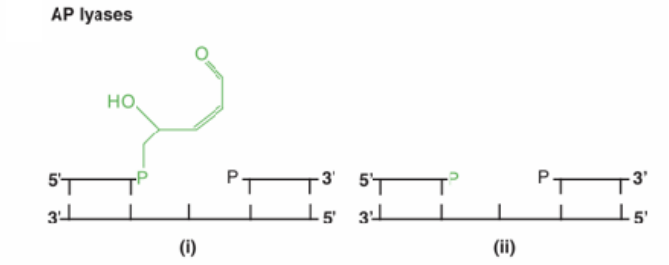

Figure 6. Types of termini generated by bifunctional glycosylases/AP lyases as a part of BER. There are two classes of the bifunctional glycosylases/AP, both generating different types of termini, firstly (i) phospho α,β-unsaturated aldehyde termini generated by human OGG1 and NTH1 and secondly (ii) 3´phosphate generated by NEIL 1 and NEIL2 (adopted and edited from Weinfeld et al., 2011).

4.2 Role of PNKP in DNA single-strand break repair

4.2.1 Detection of DNA single-strand breaks (SSBs)

The first step in SSBR is the recognition of the strand break terminus, which is done by poly(ADP-ribose) polymerases (PARPs) (Lindahl *et al.*, 1995). The human PARP1 enzyme was the first identified member of the PARP superfamily and, along with PARP2 and PARP3, plays a role in DNA strand break repair (Amé, Spenlehauer and de Murcia, 2004; Boehler *et al.*, 2011). PARP1 binds DNA by two zinc binding domains in the N-terminal region, F1 and F2, required for strand break recognition, and their mutation leads to loss of DNA strand break-dependent PARP activation (Ikejima *et al.*, 1990). The enzyme also contains another zinc domain, F3, which is not required for DNA binding but plays a role in DNA-dependent activation and contributes to chromatin modification by PARP1 (Langelier *et al.*, 2010). In addition, PARP1 contains a BRCT domain that includes the central automodification domain, which allows for auto-poly (ADP-ribosylation) (PAR), as well as a catalytic domain containing a WGR domain and ADP-ribosyl transferase (ART) (Altmeyer *et al.*, 2009). PARP2 and PARP3 lack the three zinc-binding domains and use only WGR for their catalytic activity.

Upon binding to the DNA strand break, the catalytic activity of PARP1 increases 500-fold (Simonin *et al.*, 1993). Activation of PARP leads to the synthesis of PAR chains from NAD^+ (Chambon, Weill and Mandel, 1963). The PAR chains are then bound onto PARP itself in a process called automodification (Yoshihara *et al.*, 1977) or onto other proteins such as histones in a heteromodification process (Messner *et al.*, 2010). Most of the PARylation is derived from PARP1 activity, while the remaining 10 % is generated by PARP2 (Amé *et al.*, 1999).

PARylation can lead to the recruitment of other DNA repair proteins with PAR-binding motifs. These include XRCC1, which binds to PARP1 via the XRCC1 BRCT1 domain (Masson *et al.*, 1998). However, PARP2 can also bind XRCC1 upon PARP2 PARylation, since XRCC1 requires only low amounts of ADP ribosylation for efficient recruitment to DNA damage sites (Hanzlikova *et al.*, 2017). The mammalian XRCC1 forms a constitutive complex with ligase III (LIG3) and is required for normal LIG3 levels (Caldecott *et al.*, 1994). The LIG3 gene encodes for two DNA ligase isoforms, LIG3α and LIG3β, which differ in the C-terminus sequence (Mackey et al., 1997). Interestingly, LIG3α expresses a C-terminal BRCT domain that permits the binding to the BRCT II domain present in XRCC1 (Nash *et al.*, 1997; Taylor *et al.*, 1998).

4.2.2 Processing of DNA break termini

for further processing by DNA polymerases and DNA ligases. Specific enzymes are responsible for processing unique termini chemistries, and some of these enzymes have also been shown to interact with XRCC1.

The gene APEX1 gene encodes for APE-1, which interacts with XRCC1 and converts 3′PG, one of the two typical termini caused by oxidative damage, to 3′hydroxyl termini (Winters *et al.*, 1994). The enzyme Aprataxin (APTX) binds to phosphorylated XRCC1 by an N-terminal FHA domain similar to the FHA domain present in PNKP (Date *et al.*, 2004). APTX processes termini generated by abortive DNA ligation activity, which have adenosine monophosphate on their terminal 5′ phosphate, generating 5′P termini (Ahel *et al.*, 2006).

Regarding termini generated by abortive TOP1 activity, upon the residual peptide cleavage done by TDP1, the generated 3′P are subsequently processed by PNKP, while both enzymes interact with the scaffold protein XRCC1(Plo *et al.*, 2003). Alternatively, the residual peptide can be cleaved by APE2, encoded by the APEX2 gene. APE-2 processing results in a 1-3 nucleotide single-stranded DNA gap; however, with 3′OH termini and therefore not requiring further processing for subsequent repair completion (Álvarez-Quilón *et al.*, 2020).

PNKP is responsible for end-processing both 3′P and 5′OH termini generated by various sources of SSBs. A standard model of SSBR processing is that upon PARP detection, XRCC1 binds in complex with LIG3. Subsequently, PNKP is recruited to SSBs via interaction with scaffold protein XRCC1 in complex with LIG3α, and the interaction consequently promotes the PNKP kinase and phosphatase activity (Whitehouse *et al.*, 2001). The enhancement of the PNKP enzymatic activity is proposed to be achieved by increasing the strand break discrimination capacity and displacement from processed DNA termini. The binding affinity of PNKP to 5′P termini is only five times smaller than for 5′OH termini, suggesting that PNKP does not immediately leave the termini after processing. Therefore, it is thought that XRCC1 can accelerate the displacement of PNKP from processed DNA ends. The two proposed ways the displacement can be achieved are either by competition of XRCC1 with PNKP for the 5′P, since 5′P has a 15 times higher affinity for XRCC1 than PNKP when PNKP is in complex with XRCC1, or by a steric hindrance on the 3′P and PNKP interaction exerted when PNKP is in complex with XRCC1. A similar model was also proposed for the PNKP phosphatase activity (Mani *et al.*, 2007). Recruitment of PNKP by XRCC1 has been implied by several studies, for example, by the lack of PNKP foci in XRCC1-lacking cells upon hydrogen peroxide treatment (Loizou *et al.*, 2004) or reduction of PNKP recruitment upon microirradiation in the same cell model (Hanssen-Bauer *et al.*, 2011). However, some data show that binding of PNKP to the strand break site might, on the contrary, be necessary for XRCC1 binding in case of 3′ modified termini (Parsons *et al.*, 2005). This leads to another SSB termini processing model, where PNKP binds first to the 5′OH termini at the site of the lesion, converts the termini and afterwards interacts with XRCC1, which enables faster dissociation (Mani *et al.*, 2007). Neither of the models has

been disproved, and no study yet confirms with certainty which is the correct one or whether both can be correct depending on circumstances.

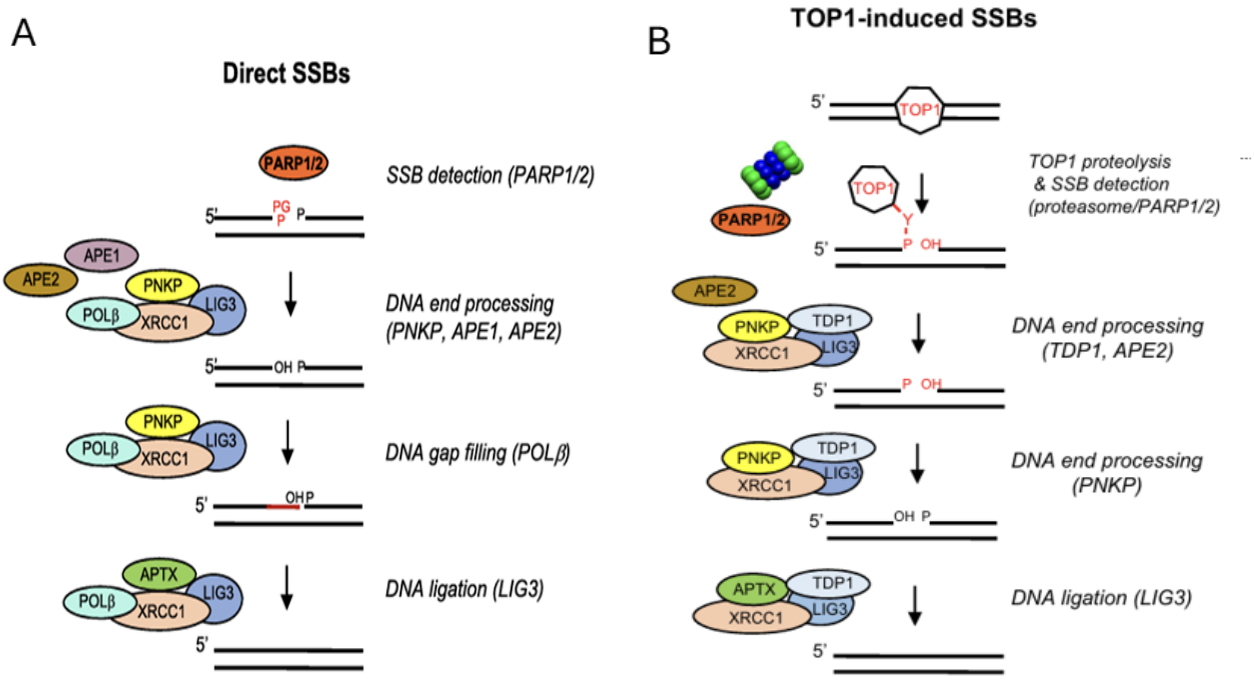

Figure7. Models of DNA single-strand break repair. A – Model of repair of SSBs caused by oxidative damage. Firstly, the breaks are detected by PARP1/2, which leads to the recruitment of the XRCC1-LIG3 complex. The n 3´P and 3´PG DNA termini, generated by the damage, are then processed. In a case of 3´P by PNKP, which also interacts with XRCC1 and in the case of 3´PG by APE1 or APE2. After the restoration of 3´OH termini, DNA gaps are filled by POLB and ligated by LIG3. If the ligation becomes abortive, APTX processes the 5´AMP termini for ligation to continue (adopted from Caldecott, 2022). B – Model for TOP1 abortive activity induced strand break repair. The TOP1cc gets degraded by the proteasome, and the break is recognised by PARP1/2, which recruits the XRCC1 complex. After the proteolysis, a peptide residue stays on the 3´ DNA termini, and it is processed either by APE2 or TDP1, which generates 3´P, which PNKP therefore processes. Lastly, the DNA is ligated by LIG3; if abortive activity occurs, the generated 5´AMP termini are processed by APTX (adopted from Caldecott, 2022).

4.2.3 Gap-filling and DNA ligation

When the canonical termini are present at the SSBs, gap-filling is performed by polymerase β (POLB), which binds to XRCC1 at the terminal N-terminal domain with its palm-thumb domain of the active site (Marintchev *et al.*, 2000). This interaction is suggested to recruit POLB to strand break sites and even slightly increase POLB level in cells (Parsons *et al.*, 2008). However, it seems that when the XRCC1 NTD domain is mutated, it has no significant impact on strand break repair after treatment with hydrogen peroxide, which causes oxidative damage. This hints at the possible involvement of other polymerases in SSBR (Breslin and Caldecott, 2009). As abovementioned, LIG3 is in a constitutive complex with

XRCC1 and is responsible for ligation upon termini processing and gap-filling. The interaction with XRCC1 stabilises LIG3, directs LIG3 to DNA damage sites and is essential for nuclear localisation (Mortusewicz, 2006). Interestingly, the lack of XRCC1 and LIG3 interaction does not severely impact cell survival upon oxidative or alkylation damage (Breslin and Caldecott, 2009). This might be due to the presence of LIG1, which can replace LIG3 activity or possibly due to the availability of an alternative repair pathway. On the contrary, in mitochondria, where LIG1 is missing, the presence of LIG3 is necessary (Gao *et al.*, 2011; Simsek, Furda, *et al.*, 2011). Interestingly, LIG3 also contains a zinc finger domain similar to the zinc finger domain present in PARP1 (Wei *et al.*, 1995). Several functional roles have been proposed for this domain, such as displacing PARP on DNA breaks, allowing repair proteins including LIG3 access to the DNA lesion (Mackey *et al.*, 1999) or a possible role in alternative non-homologous end joining (Wang *et al.*, 2005; Simsek, Brunet, *et al.*, 2011).

4.2.4 Role of PNKP in base excision repair (BER)

BER is responsible for repairing most endogenous DNA base lesions, such as those caused by alkylation, oxidation or deamination. There are two major pathways which differ in the number of excised bases during BER: short and long patch BER. In both pathways, the damaged base is recognised by one of many DNA glycosylases and excised, leaving an abasic site (Jacobs and Schär, 2012), which APE1 processes, generating a 5′deoxyribose termini (Demple, Herman and Chen, 1991).

In short patch BER, the lyase activity of POLB removes the 5′-deoxyribose terminus (Matsumoto and Kim, 1995), allowing for insertion of the missing nucleotide. This step is followed by LIG3-mediated DNA ligation, which is in complex with XRCC1 (Cappelli *et al.*, 1997). In contrast, long patch BER, POLB, or possibly polymerase δ, inserts additional nucleotides, resulting in displacement of the 5′single-strand flap that is subsequently excised by the FEN1 endonuclease (Frosina et al., 1996; Klungland and Lindahl, 1997) and then ligated by LIG1, which interacts with PCNA (Levin et al., 2000).

Some damaged bases are handled by bifunctional DNA glycosylases/lyases, which can both excise the damaged base and incise the abasic site. The two classes have already been described above. The NEIL1 and NEIL2 glycosylases act on various damaged bases, including 5-hydroxyuracil, uracil, thymine glycol and 8-oxoguanine (Wiederhold *et al.*, 2004). Interestingly, NEIL1 demonstrates comparable catalytic activity for damaged bases found in single-strand sequences and DNA containing bubbles, similar to its activity in duplex DNA. Conversely, NEIL2 prefers bubble DNA, unlike NTH1 and OGG1, which process only duplex DNA substrates. The preference for bubble DNA suggests NEILs involvement in transient transcription or replication lesion repair (Dou, Mitra and Hazra, 2003). Following base excision, NEIL 1 and NEIL2 catalyse βδ-elimination, generating 3′P termini, which are further processed by PNKP(Wiederhold et al., 2004).

Neither NEIL1 nor NEIL2 interact directly with PNKP; however, they interact with LIG3 and POLB, and NEIL2 even shows a weak interaction with XRCC1. Both LIG3 and POLB interact with XRCC1,

and since PNKP also interacts with XRCC1, these interactions are proposed to occur within a more extensive complex. These interactions suggest the overall involvement of NEIL in repair coordination, as POLB and LIG3 are involved in further gap-filling and ligation upon PNKP-processed 3´P termini into 3´OH termini (Wiederhold et al., 2004; Das et al., 2006).

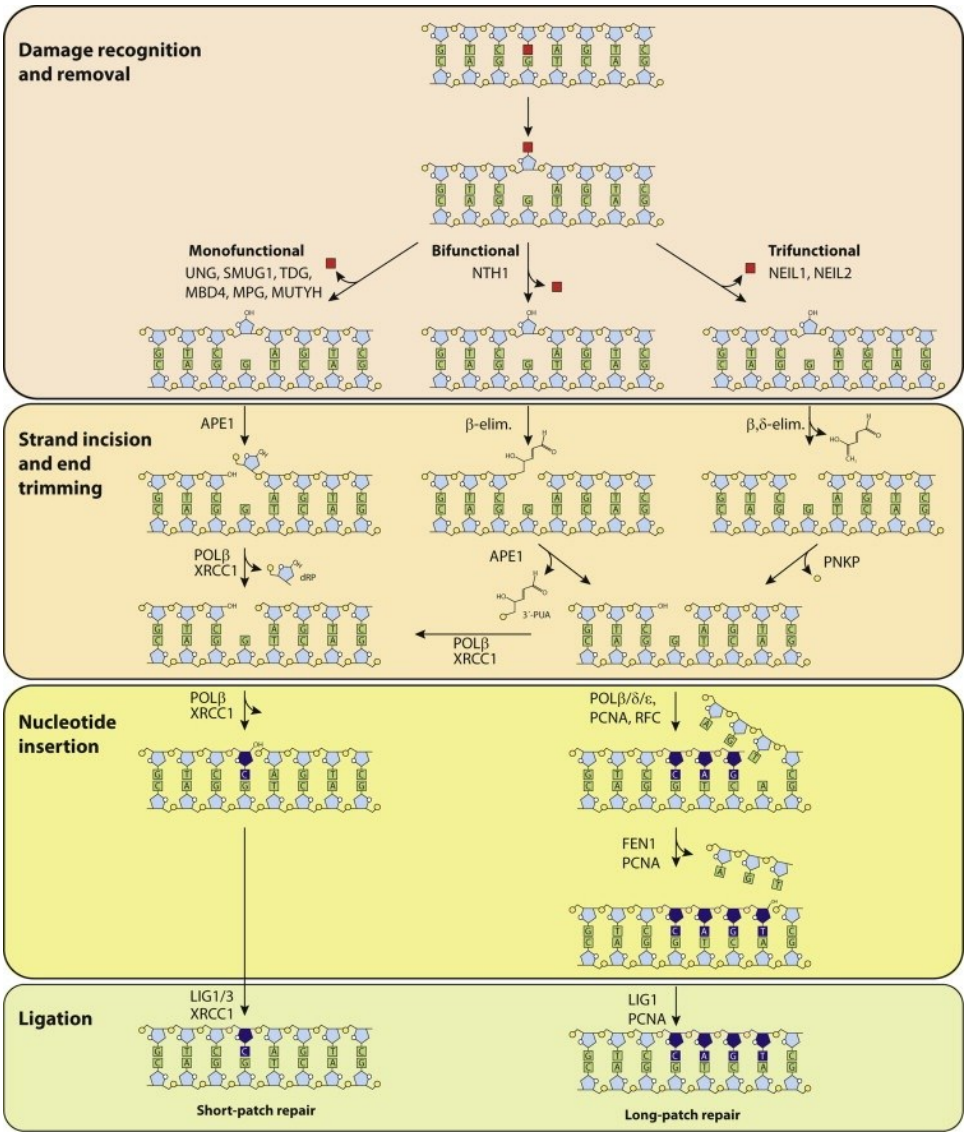

Figure 8. Model of base excision repair. Firstly, the damaged base is recognised by either monofunctional glycosylases or bifunctional glycosylase/ AP lyase. The monofunctional glycosylases excise the damaged base, leaving an abasic site, which APE1 further processes. The bifunctional glycosylases/ AP lyases can do both of these steps. There are two classes of bifunctional glycosylases/ AP lyases, differing in reaction mechanism and the types of termini they generate. If a 3´unsaturated aldehyde terminus is generated, it is further processed by APE1, and if 3´phosphate is generated, it is processed by PNKP. The further processing depends on the number of excised bases. In the short path repair, the missing base is filled by POLB, and then the DNA is ligated by LIG1 or LIG3. In the long path repair, POLB or other DNA polymerase inserts additional nucleotides, by which a 5´ single strand flap is generated and subsequently excised by FEN1 and the DNA is ligated by LIG1 (adopted and edited from Krokan et al.*, 2014)*

4.3 Role of PNKP in DNA double-strand break repair

There are three main pathways for DSBR: homologous recombination (HR), nonhomologous end joining (NHEJ) and alternative end joining. Alternative end-joining, also called microhomology-mediated end joining (MMEJ), acts as a backup pathway for NHEJ, aligning microhomologous sequences near the broken ends to facilitate end joining, which can often lead to deletions or insertions (Sfeir and Symington, 2015). NHEJ is a significant pathway in both cycling and non-cycling cells and can be active in both G1 and G2 phases; on the other hand, HR is active only in S and G2 phases, since it requires an undamaged sister chromatid to use as a repair template (Karanam *et al.*, 2012). Therefore, non-cycling cells must rely more on end joining, either the classical or alternative pathway (Heidenreich *et al.*, 2003). NHEJ is also important in V(D)J recombination, a process occurring in developing T and B cells that allows for a diverse immune response by assembling immunoglobulin and T-cell receptors (Gellert, 2002). Indeed, patients with mutations in NHEJ genes, for example, DNA ligase IV (LIG4) or XRCC4-like factor (XLF), suffer from immunodeficiency (Buck *et al.*, 2006; Altmann and Gennery, 2016). Interestingly, no patient with a PNKP mutation who also suffers from immunodeficiency has been identified, suggesting an alternative 3′P and 5′OH termini processing in V(D)J recombination.

4.3.1 PNKP in nonhomologous end-joining (NHEJ)

During NHEJ, the DSBs are recognised by the Ku70-Ku80 heterodimer, a part of the DNA-dependent protein kinase, along with the DNA protein kinase catalytic subunit, which is a serine/threonine protein kinase (Gottlieb and Jackson, 1993). The binding of Ku heterodimer is required for precise end joining, suggesting the role of Ku heterodimer as an alignment factor, increasing both efficiency and accuracy of NHEJ (Feldmann *et al.*, 2000). To bind DNA, Ku forms a ring to encircle duplex DNA, achieving high-affinity binding without the necessity for sequence specificity. Not binding a single base and almost not interacting with the sugar phosphate backbone, the DNA duplex binding is based on its fit to the minor and major grooves (Walker, Corpina and Goldberg, 2001). Once bound, the Ku heterodimer recruits the DNA protein kinase catalytic subunit (DNA-PKcs), which interacts with the carboxyl-terminal domain of Ku80. This interaction enhances the activity of the DNA-PK cs 50 to 100-fold (Singleton *et al.*, 1999).

DNA-PKcs is then phosphorylated by ATM or transphosphorylated by other DNA-PKcs, which allows for recruitment of the Artemis endonuclease to enhance termini processing. After recruitment, Artemis gets phosphorylated by DNA-PKcs, forming a complex together, allowing for cleavage of 5' and 3′ overhangs. Not all termini, however, are processed by Artemis; some of the 3′PG overhangs can be processed by TDP1, which generates 3′P that PNKP subsequently processes. Subsequently, DNA-PKcs gets autophosphorylated, which removes DNA-PKcs from the DNA damage site, therefore permitting ligation (Inamdar *et al.*, 2002; Ma *et al.*, 2002).

XRCC4 is an important scaffold protein recruited by the Ku heterodimer to the damage site, phosphorylated by DNA-PK, and stabilises the whole DNA-PK complex (Leber et al., 1998). Similarly to XRCC1, XRCC4 is tightly bound to LIG4, which is thereby also recruited and, after processing, ligates the termini (Critchlow, Bowater and Jackson, 1997). XRCC4 enhances adenylation of LIG4 and thereby LIG4 activity (Modesti, Hesse and Gellert, 1999). XLF is another protein interacting with the XRCC4-LIG4 complex, which is crucial for NHEJ, since mutations in the XLF gene lead to radiosensitivity, defects in V(D)J recombination and impaired ligation (Ahnesorg, Smith and Jackson, 2006; Buck et al., 2006). This interaction seems to regulate the activity of the complex and form XRCC4-XLF filamentous structures, which can stably bridge DNA ends (Ahnesorg, Smith and Jackson, 2006; Andres et al., 2012). As a scaffold protein, XRCC4 also binds several termini processing enzymes, such as APTX and PNKP, just like XRCC1 or Werner syndrome protein, with 3´-> 5´ helicase and endonuclease activities, possibly removing DNA-PKcs (Li and Comai, 2002; Koch et al., 2004; Imamura et al., 2023).

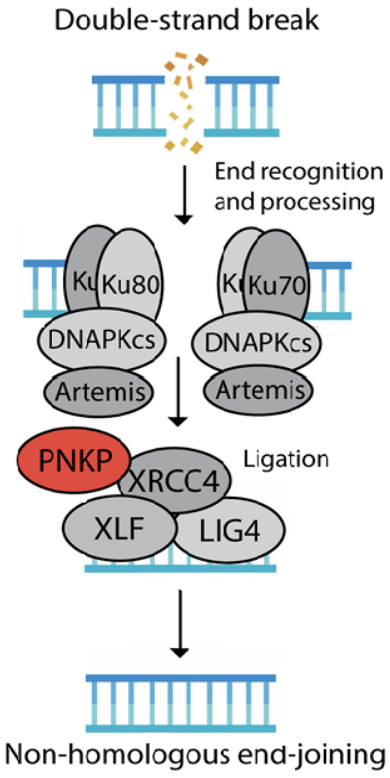

Figure 9. Model of NHEJ. Firstly, the DSBs are recognised by a Ku70-Ku80 heterodimer. When the Ku heterodimer is bound, DNA-PKcs is recruited, which allows for further recruitment of Artemis endonuclease. Artemis endonuclease then cleaves any DNA overhangs. If any 3´P or 5´OH DNA termini are present termini are present, they get processed by PNKP, which allows for DNA ligation by LIG4. LIG4 is in a complex with XRCC4 and XLF, which regulates its activity.

PNKP interacts with both phosphorylated and unphosphorylated XRCC4. If XRCC4 is unphosphorylated, the binding is relatively weak, but PNKP activity is enhanced by increasing enzyme turnover, similarly to XRCC1. In contrast, phosphorylated XRCC4 inhibits the PNKP activity, even though the binding is stronger. However, interestingly, when phosphorylated XRCC4 is in complex with

LIG4, the PNKP activity is again enhanced (Koch et al., 2004; Mani et al., 2010). About 50 % of XRCC4 in the cell is phosphorylated. Additionally, there is a more significant amount of XRCC4 than both PNKP and LIG4, suggesting that all of the mentioned possibilities of PNKP binding can simultaneously be present in the cells and potentially have specific roles during NHEJ. Mani et al (2010) proposed a model where PNKP displacement from processed termini can be achieved by unphosphorylated XRCC4 or XRCC4 in complex with LIG4. If the interaction is between unphosphorylated XRCC4 and PNKP, then phosphorylation of XRCC4 would lead to enhancement of LIG4 recruitment. However, this model still has not been directly proven, nor has it explained the role of all the binding possibilities, such as the inhibition of PNKP by phosphorylated XRCC4, if there is one.

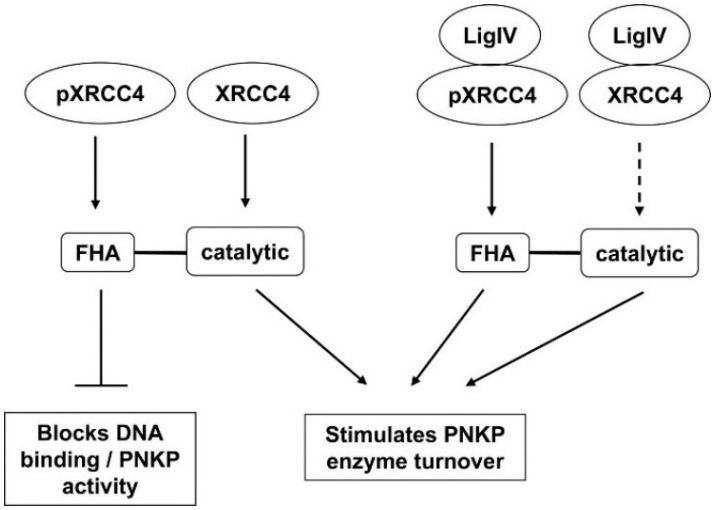

Figure 10. Scheme of the interactions between PNKP and XRCC4 and their effect on enzymatic activity. PNKP interacts with both phosphorylated XRCC4 and unphosphorylated XRCC4. When PNKP interacts with phosphorylated XRCC4, it is via the PNKP FHA domain, which recognises phosphothreonine; this interaction inhibits the catalytic activity of PNKP; however, when phosphorylated XRCC4 is also interacting with LIG4, the catalytic activity of PNKP is enhanced. Unphosphorylated XRCC4 interacts with the PNKP catalytic domain and enhances the catalytic activity of PNKP (adopted from Mani et al.*, 2010)*

4.3.2 PNKP in alternative end joining

Even though PARP1 and the XRCC1-LIG3 complex are primarily associated with single-strand break repair, they are also part of the MMEJ pathway involved in DNA double-strand break repair. The first step in MMEJ is the 5'-> 3' end resection in which the DSBs are processed by MRE11 nuclease to produce single-strand overhangs, which are recognised by PARP1. PARP1 then recruits more repair proteins, allowing the microhomologies on the single-strand overhangs to align. Subsequently, the FEN1 endonuclease removes any potential non-homologous flaps, and DNA polymerase ϑ fills the gaps. Lastly, similarly to SSBR, PARP1 recruits the XRCC1-LIG3 complex, allowing for LIG3 to ligate the broken

DNA ends (Audebert, Salles and Calsou, 2004; Sfeir and Symington, 2015). Similarly to SSBR, in case 5´OH are present at the lesion site, PNKP, which directly interacts with XRCC1, can process the DNA termini so ligation can occur (Audebert *et al.*, 2006).

4.4 Role of PNKP in mitochondrial genome maintenance

Mitochondrial DNA has a higher mutation rate and is naturally exposed to higher levels of free radicals. That is due to localisation of mtDNA near the respiratory chain and the lack of protection provided to nuclear DNA by chromatin-associated proteins, such as histones. Therefore, it must deal with more oxidative damage (Richter, Park and Ames, 1988; Kaneko and Inoue, 1998; Pesole *et al.*, 1999). However BER in mitochondria is a very important pathway in dealing with ROS-induced damage. Many of the nuclear DNA repair enzymes have also been identified in mitochondria, including APE-1 (Chattopadhyay, 2006), DNA glycosylases OGG1 and NTH1, as well as NEIL1 and NEIL2 (Hu *et al.*, 2005; Mandal *et al.*, 2012). Whenever NEIL1 or NEIL2 initiates the BER pathway, 3´ termini are generated, which must be processed for efficient ligation and completion of DNA repair. It has been shown that in mitochondria, 3´ processing occurs similarly to what happens in the nucleus, via the activity of mitochondrially localised PNKP enzyme(Mandal *et al.*, 2012). TDP1 and TOP1 have also been shown to be present in mitochondria (Das *et al.*, 2010). PNKP does not have a canonical N-terminal mitochondrial-targeting signal. However, a cryptic MTS was identified on the carboxyl-terminus, which, when mutated, impedes PNKP localisation in the mitochondria. Interestingly, PNKP also interacts with mitochondrial protein mitofilin, a transmembrane protein on the inner mitochondrial membrane, and when PNKP is depleted, mitofilin levels decrease; however, it is not apparent why (Tahbaz, Subedi and Weinfeld, 2012). In addition, upon PNKP depletion, the efficiency of both SSBR and BER in mitochondria significantly decreases, while endogenous DNA damage increases, proving the importance of PNKP in the repair process (Mandal *et al.*, 2012). Gap filling is provided by mitochondrial DNA polymerase γ (Hansen *et al.*, 2006), and subsequent ligation depends entirely on LIG3 since LIG1, which can substitute for LIG3 in the nucleus, is absent (Simsek, Furda, *et al.*, 2011).

Mitochondrial dysfunction is a hallmark of many neurodegenerative diseases, including Alzheimer´s and Parkinson´s disease (Bermúdez-Guzmán and Leal, 2019), but was also linked to a group of peripheral neuropathies called Charcot-Marie-Tooth disease (Palau *et al.*, 2009). Interestingly, a group of patients diagnosed with CMT, to be exact, CMT2B2, have a mutation in PNKP, which is possibly responsible for the given pathology (Leal *et al.*, 2018). Neurons require high amounts of ATP for their signalling function (Magistretti and Allaman, 2015); therefore, the metabolically active nature of neurons can lead to increased free radical production (Nohl and Hegner, 1978). As mentioned, it seems mtDNA is, for various reasons, more exposed to oxidative damage (Richter, Park and Ames, 1988; Kaneko and Inoue, 1998). If unrepaired damage exists, energy production could be impaired, which could lead to neuropathy (Bermúdez-Guzmán and Leal, 2019). Together, these data suggest that impairment of mtDNA repair due

to the absence or dysfunction of PNKP may be a partial cause of PNKP-related disease pathogenesis. However, a direct causal relationship has not been established so far.

4.5 PNKP in Okazaki fragment maturation

It has recently been shown that PARP1 is active not only upon encountering damage but also during normal S phase, binding to the unligated Okazaki fragment intermediates. This might be important since some Okazaki fragments might be missed and stay unligated due to the significant amount formed during the lagging strand replication (Hanzlikova *et al.*, 2018). PARP1 then binds histone PARylation factor I (HPF1), which promotes ADP-ribosylation on serine residues(Bonfiglio *et al.*, 2017; Suskiewicz *et al.*, 2020). This is essential for recruiting XRCC1 and LIG3, functioning together as a backup pathway of Okazaki fragment ligation (Kumamoto *et al.*, 2021). The canonical Okazaki processing pathway consists of polymerase δ, a nuclease, such as FEN1 and ligase I(Levin *et al.*, 1997; Maga *et al.*, 2001). Cells lacking either PARP or XRCC1 are hypersensitive to FEN1 defects, hinting at their role in the backup pathway (Hanzlikova *et al.*, 2018).

Two recent studies have suggested that PNKP is also important during replication (Mashayekhi *et al.*, 2024; Tsukada *et al.*, 2025). However, one study suggests PNKP involvement in canonical and PARP1-initiated Okazaki fragment processing (Tsukada *et al.*, 2025). In contrast, the second study specifically proposes the role in the backup pathway (Mashayekhi *et al.*, 2024). Both studies have agreed that PNKP is phosphorylated on threonine 118 by cyclin-dependent kinase 1 or 2 in a cell cycle-dependent manner, which they imply is important for recruiting PNKP to the unligated Okazaki fragments (Mashayekhi *et al.*, 2024; Tsukada *et al.*, 2025). One of the studies also implies that PNKP is necessary for the processing of unligatable 5´OH termini of OF, since when PNKP is lacking, those OF fragments with 5´OH termini accumulate in the cell (Mashayekhi *et al.*, 2024). At the same time, the second study states that while both activities are important, phosphatase activity is essential for accurate fork progression (Tsukada *et al.*, 2025). However, neither of the studies explains how the noncanonical termini are generated. Overall, both studies show some new, exciting data, even though some are quite contradictory, presenting new research opportunities to clarify the exact role of PNKP in replication, if there indeed is one.

5 The consequences of PNKP dysfunction in diseases

5.1 The role in neurological diseases

PNKP mutations have been implicated as the cause of three neurological diseases with varying severity and pathologies, from microcephaly to neurodegeneration. The most severe is microcephaly, seizures, and developmental delay (MCSZ), then ataxia with oculomotor apraxia 4 (AOA4), and the least severe is Charcot-Marie-Tooth disease 2B2 (CMT2B2). However, the severity and even pathology can differ between individual mutations.

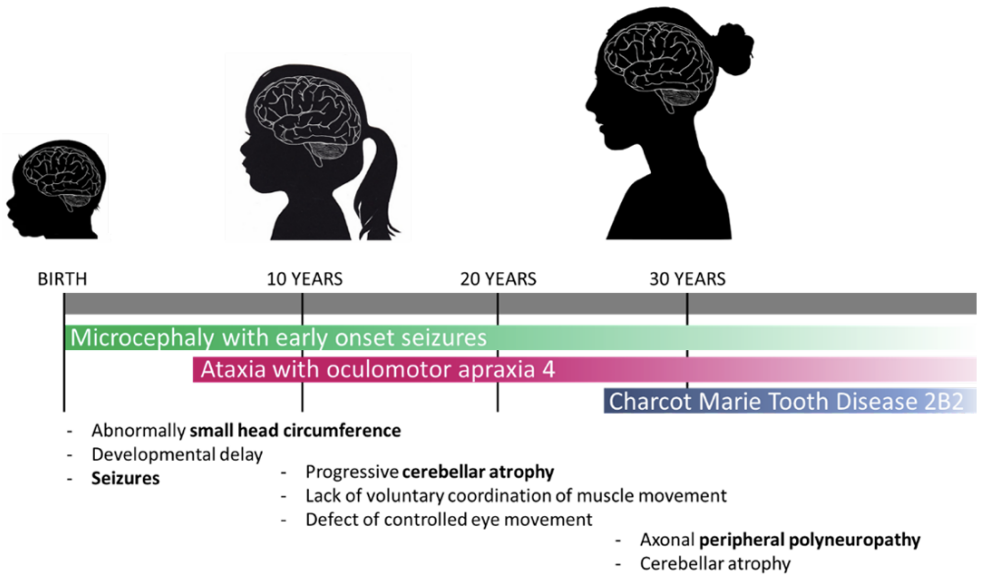

Figure 11. Scheme of onset of the PNKP-related diseases. MCSZ is usually diagnosed right after birth, with a characteristically small head circumference, and presents with developmental delay and seizures. Ataxia with oculomotor apraxia 4 is generally diagnosed during childhood, and presents with progressive cerebellar atrophy, a lack of voluntary muscle movement and defects in controlled eye movements. Charcot-Marie-Tooth disease is the mildest one and is diagnosed in adulthood, presenting with axonal peripheral polyneuropathy and cerebellar atrophy.

5.1.1 Microcephaly, seizures and developmental delay (MCSZ)

Microcephaly, seizures, and developmental delay (MCSZ) is an autosomal recessive disorder with both homozygous and heterozygous PNKP mutations, which cause varying degrees of disease severity. The MCSZ has the following pathologies: microcephaly, infantile-onset seizures, developmental delay, and various behavioural problems, such as hyperactivity. It is diagnosed after or, in some cases, even before birth (Shen *et al.*, 2010; Xie *et al.*, 2025).

The first connection between PNKP mutations and MCSZ was described in a study on seven Middle Eastern and European families presenting with disease symptoms. Three Palestinian families had a homozygous point mutation in the phosphatase domain, resulting in a nonconservative amino acid change from glutamic acid to lysine (E326K). Two more Arabic families had a homozygous 17-base pair duplication in the kinase domain (exon 14), leading to a frameshift mutation T424GfsX48. One of the European families had two heterozygous mutations: the frameshift mutation in the kinase domain (T424GfsX48) and a point mutation in the phosphatase domain resulting in an amino acid exchange from leucine to phenylalanine (L176F). The last family, also of European descent, also had heterozygous mutations: the T424GfsX48 frameshift mutation and a 17-base pair deletion in the kinase domain (intron 15), which results in impairment of proper mRNA splicing. Interestingly, this family was also less severely affected, possibly because some of the PNKP was spliced correctly (Shen *et al.*, 2010). Since

then, more mutations have been found, mainly in the kinase, some in FHA domain and two more found in the phosphatase domain (Nakashima *et al.*, 2014; Kalasova *et al.*, 2019; Marcilla Vázquez *et al.*, 2021; Jiang *et al.*, 2022; Thuresson *et al.*, 2024).

Interestingly, there were some mutations resulting not only in pathologies of MCSZ but also in ataxia, oculomotor apraxia or neuropathy, which are usually connected with AOA4 (Poulton *et al.*, 2013; Taniguchi-Ikeda *et al.*, 2018; Gatti *et al.*, 2019; Bitarafan *et al.*, 2021). Surprisingly, one of these cases even has the same T424GfsX48 frameshift mutation observed before, without the additional pathologies. However, the patients were born to consanguineous parents, implying that there are possibly more mutations leading to the pathology combination (Poulton *et al.*, 2013). Nevertheless, it shows how thin the line between the two disorders can be.

Experiments done in mice show that complete depletion of PNKP leads to embryonic lethality, suggesting a crucial role in development (Shimada *et al.*, 2015). That corroborates the observation that all patients show at least some PNKP protein levels in cell extracts, although greatly reduced in PNKP-mutated patients (Shen *et al.*, 2010; Reynolds *et al.*, 2012). Interestingly, it does not seem that the levels of PNKP correspond with the severity of the pathology (Kalasova *et al.*, 2020).

Mutations in NHEJ genes, such as LIG4, XRCC4 or XLF, generally lead to microcephaly, caused, for example, by impairment of LIG4, XRCC4 or XLF (Buck *et al.*, 2006; Murray *et al.*, 2015; Frizinsky *et al.*, 2022). However, MCSZ patient-derived fibroblasts do not have a significant decrease in DSBR, suggesting an alternative must be able to take over when PNKP is lacking or the residual PNKP activity is enough. On the contrary, SSBR is severely decreased, implying defective SSBR as the disease. Interestingly, the fibroblasts from CMTB2B patients have a lower level of SSBR defect than fibroblasts from patients with far more severe MCSZ and AOA4, implying the level of SSBR dysfunction as a possible determinant of disease severity (Kalasova *et al.*, 2020).

It is still unclear which of the two PNKP enzymatic activities is responsible for the pathology. The current data suggest that an impairment in phosphatase activity possibly causes MCSZ. In an experiment done with MCSZ, AOA4 and CMT2B2 patient cells, only MCSZ patient cells had reduced ability to repair SSBs caused by IR. Since IR generates 3´P termini, phosphatase functionality seems lower in MCSZ cells than in AOA4 and CMT2B2 cells (Reynolds *et al.*, 2012; Kalasova *et al.*, 2020). However, in an experiment with derived residual PNKP from patient cells, there is no significant difference in the phosphatase activity between the individual disorders. The explanation for that might be that a defect in the phosphatase domain may cause overall protein instability, preventing recruitment to the strand break sites (Kalasova *et al.*, 2020).

5.1.2 Ataxia with oculomotor apraxia 4 (AOA4)

Ataxia with oculomotor apraxia is a subgroup of autosomal recessive cerebellar ataxias, caused mainly by mutations in strand break repair enzymes. For example, AOA1 is caused by a mutation of aprataxin, and AOA2 is caused by a mutation of senataxin (Moreira *et al.*, 2001; Le Ber *et al.*, 2004).

AOA4 was first described by a study done on 16 individuals, of whom 12 were affected, from 8 different families in Portugal. The most common first symptom was dystonia, with the mean age of onset being 4.3 years; other common symptoms were ataxia, oculomotor apraxia, polyneuropathy, loss of motor abilities, and distal muscle weakness. In 11 individuals, brain MRIs also showed cerebellar atrophy. All of the mutations described by this study were in the kinase domain, some homozygous and some heterozygous, with the most frequent being a point mutation causing glycine to tryptophan amino acid exchange (p.Gly375Trp) (Bras *et al.*, 2015). Interestingly, one of the heterozygous mutations of one of the patients, specifically p.Thr424Glyfs*49, was also found in one of the MCSZ homozygous patients, with some symptoms of AOA4 (Bras *et al.*, 2015; Bitarafan *et al.*, 2021). More mutations have since been described, all in the kinase domain, with varying patient phenotypes (Scholz *et al.*, 2018; Rudenskaya *et al.*, 2019; Freitas, Costa and Rocha, 2021).

Other disorders resulting from SSBR deficiency, such as spinocerebellar ataxia with axonal neuropathy, which is caused by defective TDP1, also lead to neurodegeneration (Takashima *et al.*, 2002). It is therefore not a surprise that patient-derived fibroblasts show reduced SSBR rates after camptothecin treatment. Having this in mind, it was also proposed that reduced 5′ kinase activity may be the cause of neurodegeneration in PNKP-mutated disease (Kalasova *et al.*, 2020).

5.1.3 Charcot-Marie-Tooth disease type 2B2 (CMT2B2)

Charcot-Marie-Tooth disease consists of a group of heterogeneous peripheral neuropathy disorders. The first case connecting PNKP and CMT was a 17-year-old patient with sensory-motor axonal neuropathy, hammertoes and absent deep tendon reflexes, which was linked to a homozygous p.Thr408del mutation (Pedroso *et al.*, 2015). A new autosomal recessive version of CMT was reported in a large Costa Rican family presenting with axonal neuropathy, impaired deep tendon reflexes and distal muscle wasting, the age of onset being between 28 and 42 years (Leal *et al.*, 2018)

CMT2B2 was initially thought to be caused by a missense mutation in the Mediator complex 25, but is caused by PNKP, describing a point homozygous mutation p.Gln517ter. Analysis of five more non-related Costa Rican patients showed a compound heterozygous mutation p.Gln517ter/Thr408del (Leal *et al.*, 2018).

As in AOA4 and MCSZ patient fibroblasts, CMT2B2 has a defect in SSBR after camptothecin; however, this defect is to a lesser extent than that of MCSZ and AOA4 cell lines, which is consistent with CMT2B2

being the mildest of the diseases, suggesting that the level of SSBR defect corresponds with the disease severity (Kalasova *et al.*, 2020).

5.2 PNKP defects and their connection to cancer

5.2.1 Cancer patients with PNKP mutations

PNKP defects are generally not associated with elevated cancer risk; however, recently, two patients with PNKP mutations and cancer have been identified.

Firstly, an MCSZ patient with a cerebellar high-grade tumour, glioblastoma multiforme (GBM). The patient had a heterozygous PNKP mutation: a point mutation causing proline to leucine amino acid exchange (P101L) in the FHA domain and a point mutation in the phosphatase domain, which leads to a change of threonine to methionine (T323M). The P101L mutation was already described in an MCSZ patient with some AOA4 symptoms. However, the T323M phosphatase domain mutation is novel. The T323M mutation severely impacted PNKP phosphatase activity and, to a lesser extent, kinase activity and overall decreased PNKP levels. In contrast, the second mutation in the FHA domain had almost no effect on the enzymatic activity. However, it reduced XRCC4 binding affinity, even though likely not significantly, since cells with the P101L have similar DSBR to wild-type cells after complementation. Sequence analysis of the tumour showed not only mutated PNKP but also, for example, mutations leading to loss of heterozygosity in TP53 and ATRX deletion, both of which are common for GBM (Schwartzentruber *et al.*, 2012; Jiang *et al.*, 2022). Since the patient's whole genome was not sequenced, it is difficult to determine the mutation time of the different genes. However, this study proposes a theory that if the PNKP mutation preceded the others, the impairment of strand break repair could have led to the GBM-associated mutations and therefore to cancer development (Jiang *et al.*, 2022).

The second case is a patient diagnosed with epilepsy, microcephaly and mental retardation, who developed an acute myeloid leukaemia. A homozygous p.T424Gfs *49 frameshift mutation was identified, which was previously identified in a heterozygous MCSZ patient, corresponding with the patient displaying MCSZ symptoms.(Bitarafan *et al.*, 2021; Bayram *et al.*, 2022). However, this study does not describe any other mutations or mention further genome sequencing, so it is hard to conclude whether the PNKP mutation affected the development of cancer.

5.2.2 PNKP as a target for cancer treatment

Synthetic lethality is a cancer treatment method that uses mutations in two non-allelic genes, where mutation of one of the genes is not lethal, but disruption of both leads to cell death. The purpose of the method is to target cancerous cells based explicitly on already present mutations, such as a mutated tumour suppressor or an activated protooncogene, which are absent in non-cancerous cells (Kaelin, 2005; Iglehart and Silver, 2009).

A small molecule, A12B4C3, has been identified as a PNKP inhibitor, making PNKP a good choice for a synthetic lethal relationship (Freschauf *et al.*, 2009). Several proteins, such as the protein tyrosine phosphatase SHP-1, a tumour suppressor, have been identified as potentially lethal with PNKP, which could then be an effective treatment for SHP-1-depleted cancers (Mereniuk *et al.*, 2012). Another synthetic lethal PNKP partner is a tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN). A study has shown that co-depletion of PNKP and PTEN leads to apoptosis and that inhibition of PNKP in PTEN knockout cells enhances radiosensitivity. Together, these data suggest that inhibition of PNKP in PTEN-deficient tumours in combination with radiotherapy might be a potential treatment for PTEN-deficient cancers (Mereniuk *et al.*, 2013).

6 Conclusions

PNKP mutations have been implicated as the cause of neurological diseases, including MCSZ, AOA4 and CMTB2B (Shen *et al.*, 2010; Bras *et al.*, 2015; Leal *et al.*, 2018). However, the exact molecular mechanism leading to disease pathology remains unclear. A study by Kalasova *et al.* (2020) shows that patient fibroblasts exhibit defects in SSBR, but not in DSBR, which might be surprising since PNKP is involved in both. The study also suggests the level of SSBR defect as a possible determinant of the disease severity, since patients with milder disorder CMTB2B2 also have lower SSBR impairment. The lack of DSBR may be further supported by the absence of immunodeficiency in patients with PNKP mutation, as PNKP, as a part of NHEJ, participates in V(D)J recombination. This suggests that residual levels of PNKP might be sufficient for DSBR, or there might be an alternative pathway that does not require PNKP; however, there is no evidence to support either explanation. Interestingly, recent studies from Mashayekhi *et al.* (2024) and Tsukada et al. (2025) suggest that PNKP is involved in Okazaki fragment maturation (OFM). Although both studies present convincing data, their conclusions regarding the exact role of PNKP differ. While Tsukada et al. (2025) suggest the involvement of PNKP in both the conventional OFM and PARP1-initiated backup pathway, Mashayekhi *et al.* (2024) propose the role to be specifically in the backup pathway. Even though the involvement of PNKP in OFM remains uncertain, if future research confirms the current findings, a new potential cause for PNKP-related pathologies may arise.

Since PNKP is also involved in mitochondrial repair pathways (Mandal *et al.*, 2012; Tahbaz, Subedi and Weinfeld, 2012), it would be valuable to explore whether PNKP mutations impact DNA strand break repair in mitochondria. If so, how might this impact mitochondrial function, and could it also contribute to disease pathology? This is particularly interesting since mitochondrial dysfunction has been linked to neurodegenerative diseases and CMT, although not specifically to CMTB2B (Palau *et al.*, 2009; Bermúdez-Guzmán and Leal, 2019).

Understanding the structure of PNKP and the mechanism of the PNKP DNA kinase and phosphatase enzymatic activities is crucial for fully comprehending its functions in DNA strand break repair. Both DNA substrate binding to the individual catalytic domains and subsequent enzymatic processing have been described in *in vitro* studies, which, however, can never fully simulate the exact cellular conditions. Structural studies have vast potential to predict how individual mutations in patients may impact substrate binding, catalytic activity and overall protein stability in cells, which has not yet been described. These substrates could also provide insight into disease pathogenesis.

One structural study by Dobson and Allinson (2006) describes the precedence of PNKP´s DNA 3´P phosphatase activity over its 5´kinase activity and the overall higher efficiency of the phosphatase domain, supporting the theory that phosphatase activity may be more important. The higher evolutionary conservation of the PNKP DNA phosphatase domain compared to the DNA kinase domain ;also supports

this hypothesis. Additionally, the study by Kalasova *et al*. (2020) suggests that dysfunction in PNKP´s 3´P phosphatase domain could contribute to neurodevelopmental impairment, a symptom of MCSZ, the most severe of the PNKP-related diseases. Kalasova *et al*. (2020) propose that the reason may not be the lack of the DNA 3´P enzymatic activity itself, but rather protein destabilisation in the patient cells.

Most mutations in the *PNKP* gene linked to disease occur in the kinase domain, with only four mutations identified in the phosphatase domain (Nakashima *et al.*, 2014; Kalasova *et al.*, 2019; Marcilla Vázquez *et al.*, 2021; Jiang *et al.*, 2022; Thuresson *et al.*, 2024). Interestingly, the patients with phosphatase domain mutations still have residual PNKP levels, which aligns with findings from Shimada et al. (2015), who showed that complete PNKP depletion is embryonically lethal in a mouse model. Shimada *et al.* (2015) also presents data suggesting the necessity of PNKP in neurodevelopment. If impaired phosphatase activity impacts the protein stability, it is possible that more mutations of the PNKP phosphatase domain occur, leading to greater destabilisation and lower PNKP levels. However, these mutations might not be detected because they are embryonically lethal. Kalasova *et al.* (2020) also propose that defects in kinase activity lead to neurodegeneration. In summary, the current theory suggests that impairment of phosphatase activity leads to PNKP destabilisation, resulting in lower levels of PNKP and neurodevelopmental dysfunction, as observed in MCSZ. In contrast, impairment of kinase activity leads to neurodegeneration, as seen in AOA4 and CMTB2B patients. However, given the wide variety of phenotypes between patients, and sometimes even combinations of pathologies, it is possible that other factors may influence the pathology of individual mutations. Notably, two patients with PNKP mutations have recently been diagnosed with different types of cancer (Bayram *et al.*, 2022; Jiang *et al.*, 2022). Although it is uncertain whether the PNKP mutations played a role, this raises the possibility of their involvement in cancer development.

Overall, there remains considerable uncertainty surrounding PNKP, with new questions continually emerging. With access to some yet-unpublished data and the proposal of a novel approach to studying the effects of PNKP depletion, which could help answer some of these questions, it seems that PNKP research has an exciting future.

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