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Neurological disorders associated with DNA strand-break processing enzymes

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Abstract

The termini of DNA strand breaks induced by reactive oxygen species or by abortive DNA metabolic intermediates require processing to enable subsequent gap filling and ligation to proceed. The three proteins, tyrosyl DNA-phosphodiesterase 1 (TDP1), aprataxin (APTX) and polynucleotide kinase/phosphatase (PNKP) each act on a discrete set of modified strand-break termini. Recently, a series of neurodegenerative and neurodevelopmental disorders have been associated with mutations in the genes coding for these proteins. Mutations in *TDP1* and *APTX* have been linked to Spinocerebellar ataxia with axonal neuropathy (SCAN1) and Ataxia-ocular motor apraxia 1 (AOA1), respectively, while mutations in *PNKP* are considered to be responsible for Microcephaly with seizures (MCSZ) and Ataxia-ocular motor apraxia 4 (AOA4). Here we present an overview of the mechanisms of these proteins and how their impairment may give rise to their respective disorders.

Keywords

Tyrosyl DNA-phosphodiesterase 1; DNA repair; Aprataxin; DNA strand breaks; Polynucleotide kinase/phosphatase; Neurodegeneration

1. Introduction

DNA strand breaks continually arise in cells. The primary sources for this damage are endogenously produced reactive oxygen species (ROS). However, other factors can also contribute to strand break induction. These include DNA metabolic processes, such as DNA repair, topoisomerase-catalyzed DNA unwinding or DNA ligase-catalyzed strand rejoining, that can be aborted prior to completion (Andres et al., 2015; Caldecott, 2008). The maintenance of the genome is therefore dependent on DNA repair pathways that respond to single- and double-strand breaks. Since the chemical makeup of the termini at many of these strand breaks precludes simple religation, a key component in the repair process is the restoration of the strand break chemistry to a form suitable for DNA polymerases and

ligases, i.e. 3'-hydroxyl and 5'-phosphate termini (Andres et al., 2015). Several enzymes are now known to each act on a subset of strand-break termini including tyrosyl DNA-phosphodiesterase 1 (TDP1), aprataxin (APTX) and polynucleotide kinase/phosphatase (PNKP) (Pommier et al., 2014; Schellenberg et al., 2015; Weinfeld et al., 2011).

In addition to the roles these enzymes might play in preventing carcinogenesis and response to cancer therapy, recent studies have highlighted their importance in protection against neurological disorders. Because of their relatively long life span, neuronal cells may be especially sensitive to the deleterious consequences of endogenous DNA damage. Mutations in the genes coding for each of these enzymes are responsible for rare autosomal recessive diseases with varying degrees of severity. A mutation in *TDP1* gives rise to the neurodegenerative disorder Spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima et al., 2002), while mutations in *APTX* are responsible for another neurodegenerative disorder, Ataxia-ocular motor apraxia 1 (AOA1) (Moreira et al., 2001). Mutations in *PNKP* underlie both a neurodevelopmental disorder, Microcephaly with seizures (MCSZ) (Shen et al., 2010) and a neurodegenerative disease, Ataxia-ocular motor apraxia 4 (AOA4) (Bras et al., 2015). Here we describe each of these enzymes including the DNA lesions that they act on, their mechanism of action, their role in DNA repair pathways in the nucleus and mitochondria, and how the disease-associated mutations impair their function and cause the observed neurological pathology.

2. Spinocerebellar ataxia with axonal neuropathy (SCAN1) and TDP1

SCAN1 is a neurological autosomal recessive disorder characterized by late childhood (13–15 years of age) onset of a series of symptoms starting with slowly progressing cerebellar ataxia causing unsteady gait, which ultimately leads to wheelchair dependency, followed by areflexia (loss of reflexes) and peripheral neuropathy. Additional symptoms include involuntary movements of the eye (gaze nystagmus) and slurred speech (cerebellar dysarthria). However, affected individuals retain normal intellect and lifespan with no indication of a higher incidence of cancer. Extra-neurological features of SCAN1 include hypoalbuminemia and hypercholesterolemia. SCAN1 is caused by a mutation of *TDP1*, the gene that encodes tyrosyl-DNA phosphodiesterase 1 (TDP1). To date only one mutation in *TDP1* has been linked to the disorder. The mutation results in an amino acid residue change (His493Arg) that disrupts a key active site of the enzyme (Davies et al., 2002; Takashima et al., 2002).

2.1. Tyrosyl-DNA phosphodiesterase 1

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is involved in the repair of several DNA lesions either as the primary agent or in a backup capacity. It has a major role in processing specific types of DNA strand breaks in the nucleus and mitochondria and has more recently been implicated in the repair of abasic sites and ribonucleosides misincorporated into DNA (Andres et al., 2015; Pommier et al., 2014).

Tdp1 was originally isolated from *Saccharomyces cerevisiae* as an enzyme that hydrolyzed the bond between topoisomerase 1 (Top1) and DNA (Pouliot et al., 1999; Yang et al., 1996). During the course of its action Top1 incises one strand of the DNA by forming a transient

covalent bond between a tyrosine residue (Y723 in human topoisomerase 1) and the 3'-phosphate terminus of the cleaved DNA (Champoux, 1977). Following relaxation of the DNA, the enzyme normally ligates the DNA. However, poisons such as camptothecin, or its clinical derivatives irinotecan and topotecan, can stall the process by preventing the second step and preserving the protein-DNA complex, often termed the Top1 "dead-end" or Top1 cleavage complex (Hsiang et al., 1985; Pommier et al., 2010). The removal of the "dead-end" complex is achieved by partial proteolysis of the trapped topoisomerase followed by Tdp1 cleavage of the residual peptide-DNA bond (Debethune et al., 2002; Interthal and Champoux, 2011). Processing by Tdp1 occurs in two steps (Fig. 1A); Tdp1 first displaces the Top1 fragment to form a covalent Tdp1-DNA intermediate, and then catalyzes the hydrolysis of the Tdp1-DNA bond (Davies et al., 2003; Raymond et al., 2004). The second step is driven by His493, which is the altered residue responsible for SCAN1. Because the Tdp1-incised DNA retains 3'-phosphate and 5'-hydroxyl termini, processing by PNKP is required before ligation can occur (Plo et al., 2003).

Importantly from the perspective of neurological damage, other, endogenous factors can also lead to stalling of topoisomerase 1. Preexisting lesions in the DNA in close proximity to the sites of topoisomerase 1-mediated incision serve as the main source preventing DNA religation by the enzyme. These lesions include abasic sites, mismatches, nicks or gaps opposite the top1 cleavage site, and certain DNA oxidative base lesions such as 7, 8-dihydro-8-oxoguanine and 5-hydroxycytosine (Pourquier et al., 1997a,b, 1999). In addition to preventing religation, 7, 8-dihydro-8-oxoguanine and 5-hydroxycytosine in certain positions in close proximity to the topoisomerase cleavage site can also enhance DNA scission by the enzyme (Pourquier et al., 1999).

The action of Tdp1 is not confined to Top1 dead-end complexes or to single-strand breaks (SSBs). It can release a variety of 3'-adducts including 3'-terminal abasic sites (Interthal et al., 2005a) and phosphoglycolates, which are strand-break termini generated by ROS and ionizing radiation (Inamdar et al., 2002; Zhou et al., 2009). While Ape1 efficiently removes 3'-phosphoglycolates from SSBs, Tdp1 plays a critical role in their removal at double-strand breaks (DSBs) by hydrolyzing the glycolic acid function. Tdp1 also displays 3'-exonuclease activity. It can remove terminal deoxy- and ribonucleotides leaving a 3'-phosphate terminus (Interthal et al., 2005a). The importance of this function has yet to be fully explored. TDP1 has also been shown to have AP endonuclease activity, and may act in a backup capacity for APE1 (Lebedeva et al., 2013, 2011). A role for TDP1 in the nonhomologous end-joining pathway (NHEJ) for double-strand break repair (DSBR) has been proposed based on the observation that it physically interacts with XLF in a TDP1-XLF-DNA complex, which stimulates TDP1 activity at DSBs (Heo et al., 2015). These investigators also observed that TDP1 promotes DNA binding by Ku70/80.

There is mounting evidence that TDP1 may act on stalled topoisomerase 2 cleavage sites, although this is still an area of controversy. Topoisomerase 2 cleaves both strands of the DNA through the formation of covalent phosphotyrosine bonds with the 5'-termini of the incised duplex DNA, thereby allowing changes to DNA topology prior to catalyzing DNA religation by the topoisomerase (Roca and Wang, 1994). As with Top1, the action of Top2 can be inhibited at the DNA cleavage stage by chemotherapeutic agents, such as etoposide

(Pommier et al., 2010), and by preexisting abasic sites and nicks in the Top2 recognition sequence (Wilstermann and Osheroff, 2001). The primary pathway for the repair of these lesions involves 5'-tyrosyl phosphodiesterase activity of TDP2, which hydrolyzes the bond between the 5'-phosphate and Top2, followed by nonhomologous end-joining (Cortes Ledesma et al., 2009; Gomez-Herreros et al., 2013). Biochemical analysis using a variety of substrates bearing 5'-phosphotyrosyl termini showed that purified human TDP1 was capable of hydrolyzing the tyrosine residue from a substrate bearing a 4-base overhang, which resembles the break induced by Top2, but not a blunt-ended substrate (Murai et al., 2012). Tdp1-depleted and overexpressing cells have provided mixed answers. Cells from Tdp1 knockout mice and MEFs showed no hypersensitivity to etoposide (Hirano et al., 2007b), while down-regulation of TDP1 in HeLa cells did confer hypersensitivity (Borda et al., 2015). Furthermore, overexpression of TDP1 in HEK293 cells induced resistance to etoposide (Barthelmes et al., 2004).

2.2. Mitochondrial TDP1

Following up on the observation of a prominent TDP1 signal in the cytoplasm of some human neurons (Hirano et al., 2007b), Das et al. showed that a fraction of cellular human TDP1 localizes in the mitochondria (Das et al., 2010). Further analysis by Das et al. indicated that the mitochondrial protein appears to have a similar, if not identical, molecular weight as the nuclear protein, suggesting that mitochondrial TDP1 does not require post-translational modification, such as the N-terminal truncation seen with mitochondrial APE1 (Chattopadhyay et al., 2006). Mitochondrial TDP1 possesses the same biochemical activities as the nuclear protein and together with mitochondrial DNA ligase III plays a critical role in the base excision repair pathway in response to oxidative damage of mitochondrial DNA, and in the maintenance of mitochondrial DNA integrity in vivo (Das et al., 2010). Subsequently, an extensive histological analysis of human and mouse tissue revealed that Tdp1 is present in the nuclei and mitochondria of many tissues, although interestingly, only cytoplasmic TDP1 was observed in human skeletal muscle (Fam et al., 2013). This group also observed a dramatic increase in the cytoplasmic level of TDP1 in cultured human dermal fibroblasts in response to oxidative stress elicited by treatment with menadione or hydrogen peroxide (Fam et al., 2013).

2.3. SCAN1 cells and model organisms

At the DNA level, a single mutation of the *TDP1* gene (1478A > G) is the cause of SCAN1. Importantly, the resulting His493Arg alteration in TDP1 from SCAN1 patients (Fig. 2), prevents the second step in the processing of the Top1-cleavage complex by TDP1, i.e. the self-catalyzed hydrolysis of the TDP1-DNA complex, and as a consequence TDP1 remains bound at the 3'-terminus of the strand break (Interthal et al., 2005b). SCAN1 cells are defective in the repair of transcription-dependent topoisomerase I cleavage complexes (Miao et al., 2006) and single strand-break repair (SSBR) (El-Khamisy et al., 2005), as well as the removal of phosphoglycolates at DSBs (Akopiants et al., 2015; Zhou et al., 2005). Interestingly, in contrast to its displacement of Top1 and aberrant formation of a TDP1-DNA complex when handling Top1-DNA complexes, the failure of the mutant TDP1 to process phosphoglycolates is not due to the formation of a stalled TDP1-DNA complex, but rather an inability to initially displace the glycolate group (Hawkins et al., 2009; Zhou et al., 2005).

Tdp1 has been knocked out in several organisms in order to define the role of Tdp1 at the cellular level and model various facets of SCAN1. Three groups generated *Tdp1* knockout mice (Hawkins et al., 2009; Hirano et al., 2007b; Katyal et al., 2007). All reported that cells derived from these *Tdp^{-/-}* mice display a greatly reduced capacity to remove Top1-cleavage complexes. Hawkins et al. observed a failure of extracts of their *Tdp1^{-/-}* cells to remove phosphoglycolates from double-stranded substrates (Hawkins et al., 2009), but, perhaps surprisingly, neural cells derived from *Tdp1^{-/-}* mice failed to show any measurable defect in DSB repair in response to ionizing radiation (Katyal et al., 2007). None of the groups observed any overt physiological or behavioural differences between the *Tdp1^{-/-}* mice and their wild-type littermates including their CNS system. One study did note a slow progressive reduction of the cerebellum with age in the *Tdp1^{-/-}* mice (Katyal et al., 2007), but this was not observed in the knockout mice generated by Hawkins et al. even after 22 months (Hawkins et al., 2009). In particular, the latter group found that the granule and Purkinje cells in the cerebellum were present in equal numbers and appeared morphologically similar in the brains of the wildtype and *Tdp1^{-/-}* mice. The failure of the knockout mice to fully recapitulate SCAN1, particularly the failure to develop ataxia, was attributed to the difference between the obstruction to alternative repair processes imposed by the H493R Tdp1-DNA complex compared to the complete loss of Tdp1 (Hawkins et al., 2009; Hirano et al., 2007b).

Studies of *glai* (*gkt*), the *Drosophila melanogaster* ortholog of *Tdp1* resulted in starker inter-laboratory differences than observed with *Tdp1* knockout mice phenotypes (Dunlop et al., 2004; Guo et al., 2014). The earlier report indicated that loss of *gkt* severely impairs neuronal development (Dunlop et al., 2004), while the more recent study noted a comparatively benign phenotype (only in mutant females) consisting of shortened lifespan and a late onset reduced climbing ability, both of which could be rescued by expression of *TDPI* (Guo et al., 2014). The reasons for the divergent phenotypes have yet to be resolved.

2.4. Mechanism underlying SCAN1 neuronal atrophy

The question remains as to why inactivation of TDPI gives rise to the neurological symptoms of SCAN1. The question can be broken down into two components. First, why would neuronal cells be the major target, and second, what is the underlying mechanism responsible for their demise when TDPI is mutated?

SCAN1 is a neurodegenerative disorder, rather than a neurodevelopmental disorder, and is therefore considered a disease of differentiated post-mitotic neuronal cells. Several key features of neuronal cells may make them especially susceptible to death arising from endogenous damage including (i) their longevity, (ii) their high energy requirement and therefore elevated generation of oxidative free radicals (Bolanos, 2016), (iii) their high transcriptional activity, which would necessitate increased processing by topoisomerases, and (iv) unlike most other non-cycling cells neurons have a capacity to regenerate (Steward et al., 2013). Based on these properties of neuronal cells a number of possible explanations for their degeneration in SCAN1 have been put forward. Not surprisingly, most are predicated on an inability to fully repair DNA strand breaks, but a key issue has been the mechanism for formation of DSBs in non-replicating cells and the subsequent signaling

leading to cell death. One source for the induction of DSBs in neurons is transcription arrest by Top1-cleavage complexes (Sordet et al., 2010, 2009), which instigates the formation of triple-stranded DNA/RNA hybrid structures termed R-loops (Aguilera and Garcia-Muse, 2012). These DSBs activate ATM (Cristini et al., 2016; Sordet et al., 2009). While ATM is normally considered protective, it has been shown that in response to damage by camptothecin, ATM in post-mitotic neurons is phosphorylated by Cdk5 at Ser794, promoting further ATM activity that triggers cell death, possibly as a result of re-entry of the neurons into the cell cycle (Tian et al., 2009). Support for this proposed mechanism has been provided from studies with *Schizosaccharomyces pombe*. Like SCAN1 neuronal cells, quiescent *tdp1* mutated *S. pombe* cells accumulate strand breaks and undergo cell death over a period of several days through ATM/Tel1-dependent nuclear DNA degradation (Arcangioli and Ben Hassine, 2009; Ben Hassine and Arcangioli, 2009). In addition, since cell death of the *tdp1 S. pombe* mutants is not dependent on top1 function, it implies that the toxic DNA lesions are most likely strand breaks caused by reactive oxygen species (Ben Hassine and Arcangioli, 2009). This led the authors to propose that mitochondrial respiration is responsible for the neuronal cell death in SCAN1 patients. Since the mitochondrial form of TDP1 appears to be identical to the nuclear protein (Das et al., 2010), the mitochondrial TDP1 in SCAN1 is also mutated, which raises the possibility that mitochondrial DNA damage contributes to SCAN1 (Sykora et al., 2012).

3. Ataxia-ocular motor apraxia 1 (AOA1) and APTX

In 1988, Aicardi et al. reported a novel neurological syndrome in 14 patients from 10 families (Aicardi et al., 1988). The syndrome, whose symptoms included progressive ataxia, choreoathetosis (irregular movements such as twisting and writhing) and ocular motor apraxia (defective control of eye movement), was termed Ataxia-ocular motor apraxia (AOA). Since then four distinct AOA disorders have been identified, each associated with mutations in different genes: AOA1 is linked to mutations in APTX (Aprataxin) (Date et al., 2001; Moreira et al., 2001), AOA2 is linked to mutations in SETX (Senataxin) (Moreira et al., 2004), AOA3 is linked to mutations in PIK3R5 (Phosphoinositide-3-kinase regulatory subunit 5) (Al Tassan et al., 2012), and AOA4 is linked to mutations in PNKP (Polynucleotide phosphatase/kinase) (Bras et al., 2015).

The mean age of onset of AOA1 is 4.3 years and additional symptoms include cerebellar atrophy, hypoalbuminemia and hypercholesterolemia. Unlike SCAN1, for which only a single mutation has been observed in TDP1, over 20 distinct mutations in APTX have been identified in different AOA1 individuals (Fig. 2), including base substitution and frameshift mutations, and there is some evidence that the nature and site of the mutation may influence the severity of the disorder and age of onset (Le Ber et al., 2003). For example, the A198V mutation is associated with a more choreic phenotype (Le Ber et al., 2003).

3.1. Aprataxin

Following from an observation that APTX possesses an AMP-lysine hydrolase activity (Seidle et al., 2005), Ahel et al. discovered a role for APTX in the resolution of stalled DNA ligation intermediates that contain an adenosine monophosphate moiety covalently linked to

the 5'-phosphate terminus of the strand break (Ahel et al., 2006). These intermediates can arise when DNA lesions, such as 8-oxoguanine or an abasic site, at the 3'-terminus of the strand break prevent the second step of the ligation process, which involves phosphodiester formation between the DNA 3'-OH and 5'-phosphate termini and displacement of the AMP (Harris et al., 2009). Under normal circumstances, however, these lesions are considered to be a rare occurrence, but recent evidence has shown that 5'-adenylated termini frequently arise at ribonucleotide terminated strand breaks induced by RNaseH2 incision at sites of ribonucleotide misincorporation into DNA (Tumbale et al., 2014). APTX may also have the capacity to act on 3'-phosphate and 3'-phosphoglycolate termini (Takahashi et al., 2007), but this requires further validation.

APTX is composed of three major domains: an N-terminal forkhead associated (FHA) domain, and a histidine triad (HIT) domain linked to a C-terminal Cys₂His₂ Zn-finger (Znf) domain, which together comprise the catalytic domain and DNA interaction scaffold (Kijas et al., 2006; Rass et al., 2007a; Tumbale et al., 2011). The FHA domain interacts with phosphorylated XRCC1 and XRCC4 (Ahel et al., 2006; Clements et al., 2004; Sano et al., 2004) and PARP (Gueven et al., 2004; Harris et al., 2009), and mediates recruitment of APTX to DNA SSBs (Harris et al., 2009; Hirano et al., 2007a). The finding that the FHA domain also interacts with mediator of DNA-damage checkpoint protein 1 (MDC1) suggests that APTX is involved in the repair of a subset of DSBs (Becherel et al., 2010), in agreement with the observation that APTX hydrolyzes adenylated DSBs in vitro (Rass et al., 2007a). APTX acts by initially displacing the adenylate moiety from the 5'-terminus through nucleophilic attack by the His260 residue to generate a transient covalent protein-AMP complex, which is then hydrolyzed to release the free enzyme and AMP (Fig. 1B) (Rass et al., 2008; Tumbale et al., 2014).

3.2. Mitochondrial APTX

At least one isoform of APTX, containing an N-terminal mitochondrial targeting sequence, has been shown to localize to mitochondria (Sykora et al., 2011). Knockdown of *APTX* expression resulted in mitochondrial dysfunction as indicated by altered ROS production, reduced citrate synthase activity, and lower mitochondrial DNA copy number (Sykora et al., 2011). The knockdown cells also accumulated a significantly higher level of strand breaks in their mitochondrial DNA than in their nuclear DNA (Sykora et al., 2011). The possibility that mitochondria, in comparison to nuclei, do not possess an efficient backup pathway to handle adenylated-DNA termini in the absence of APTX was recently confirmed (Akbari et al., 2015).

3.3. AOA1 cells and APTX knockout mice

To date, the *APTX* mutations found in AOA1 patients are distributed in the HIT and Znf domain, including nonsense truncations, frameshifts and missense mutations, as shown in Fig. 2. No mutations linked to AOA1 have been found in the FHA domain. Using their assay for lysine-AMP hydrolysis, Seidle et al. examined several of the *APTX* mutations found in AOA1 (Seidle et al., 2005) and found that the A198V, P206L, V263G, D267G, 689insT, W279X, and 840delT proteins to be functionally inactive, while the W279R protein retained

a very low level of activity and the K197Q protein was more active, possibly reflecting its milder phenotype (Tranchant et al., 2003).

Cells derived from AOA1 patients have been widely used to study the cellular role of APTX in response to a variety of genotoxic agents, sometimes with seemingly contradictory results. There is little evidence for sensitivity of AOA1 cells to ionizing radiation (Clements et al., 2004; Gueven et al., 2004; Moreira et al., 2001; Mosesso et al., 2005), although APTX colocalizes with XRCC1 and MDC1 along tracks induced by high LET radiation (Becherel et al., 2010; Gueven et al., 2004). Several studies indicated that AOA1 cells are modestly hypersensitive to hydrogen peroxide (Clements et al., 2004; Gueven et al., 2004) and methyl methanesulfonate (MMS) (Clements et al., 2004) consistent with a role in base excision and/or SSB. However, AOA1 cells bearing the 892C > T (Gln298X) nonsense mutation do not display hypersensitivity to hydrogen peroxide or MMS, but in both cases the repair of SSBs is much slower than in normal cells, suggesting that alternative repair pathways prevent toxicity by these agents (Crimella et al., 2011). This has been substantiated by recent findings showing that DNA polymerase β can remove 5'-adenylated-deoxyribose phosphate groups through its lyase activity and that the long-patch SSB enzyme flap endonuclease (FEN1) can also remove this end group together with one or two additional nucleotides (Caglayan et al., 2014, 2015). Daley et al. similarly suggested that long patch repair may serve as a backup pathway for removal of 5'-adenylated strand-break termini based on their observation that combined loss of *HNT3* (Aprataxin homolog) and *rad27* (FEN1 homolog) in *S. cerevisiae* induces synergistic sensitivity to hydrogen peroxide and MMS (Daley et al., 2011).

Aptx^{-/-} mice show no overt phenotype (Ahel et al., 2006), although murine *Aptx*^{-/-} primary neural astrocytes respond similarly to genotoxic agents as AOA1 human cells and depend on long-patch SSB as a back up to the loss of Aptx (Reynolds et al., 2009). Murine *Aptx*^{-/-} embryonic fibroblasts (MEFs) cultured over 2 months showed reduced population doubling and a progressive increase in senescence in comparison to wild-type MEFs, which was ascribed to a progressive build up of DNA damage (Carroll et al., 2015). In order to augment the chances of observing a more obvious phenotype, Carroll et al. generated an *Aptx*^{-/-} mouse expressing a mutant form of superoxide dismutase (*SOD1*^{G93A}) thereby reducing cellular antioxidant homeostasis to increase the constitutive level of oxidative DNA damage (Carroll et al., 2015). Although this model still did not recapitulate the neurological symptoms of AOA1, spinal cord sections from the *APTX*^{-/-}*SOD1*^{G93A} mice exhibited a reduction in motor neuron survival compared with sections from *SOD1*^{G93A} mice. Interestingly, the *APTX*^{-/-}*SOD1*^{G93A} mice also displayed down-regulation of insulin-like growth factor 1 indicative of premature aging. The *APTX*^{-/-}*SOD1*^{G93A} MEFs were slightly more sensitive to hydrogen peroxide than the *APTX*^{-/-} or *SOD1*^{G93A} MEFs and showed slower strand break repair.

3.4. Mechanism underlying AOA1

This still remains an open area of research, in part because of the lack of a suitable animal model. At the molecular level, most attention has focused on the idea of a progressive accumulation of DNA lesions due to mutations in the catalytic/DNA interacting domain of

4.1. Polynucleotide kinase/phosphatase

PNKP is a bifunctional enzyme that catalyzes the phosphorylation of 5'-hydroxyl termini and the dephosphorylation of 3'-phosphate termini (Fig. 1C) (Jilani et al., 1999; Karimi-Busheri et al., 1999; Pfeiffer and Zimmerman, 1982). It is a nuclear and mitochondrial protein involved in SSBR, NHEJ and Alt-NHEJ (Audebert et al., 2006; Chappell et al., 2002; Karimi-Busheri et al., 1998; Whitehouse et al., 2001), but is not required for homologous recombination (Karimi-Busheri et al., 2007; Shimada et al., 2015). Strand breaks with 5'-hydroxyl and 3'-phosphate termini are commonly produced by hydroxyl radicals and ionizing radiation (Dedon, 2008; Henner et al., 1983), and they are also the product of Top1 dead-end complexes following removal of the covalently bound Top1 by proteolysis and the action of Tdp1 (Plo et al., 2003). In addition, strand breaks with 3'-phosphate termini are generated after removal of the glycolate moiety from phosphoglycolate termini at DSB termini by TDP1 (Inamdar et al., 2002; Zhou et al., 2009), and by several DNA glycosylases, such as NEIL1 and 2, that cleave abasic sites by β,δ -elimination (Hazra et al., 2002a,b; Rosenquist et al., 2003). The capacity of these glycosylases to act on abasic sites provides an alternative base excision repair pathway to the canonical APE1-dependent mechanism (Wiederhold et al., 2004). Down-regulation of *PNKP* expression increases spontaneous mutation frequency, indicating the importance of PNKP in protecting the genome following endogenous DNA damage (Rasouli-Nia et al., 2004). PNKP depletion also increases the sensitivity of cells to ionizing radiation, hydrogen peroxide and the topoisomerase 1 poison camptothecin (Rasouli-Nia et al., 2004).

PNKP is composed of three domains, an FHA domain at the N-terminus, similar to the APTX FHA domain, and the phosphatase domain and C-terminal kinase domain that together constitute the catalytic domain (Bernstein et al., 2005). The FHA domain is not required for enzyme activity, but, as with the APTX FHA domain, it binds to phosphorylated XRCC1 and XRCC4 for optimal response in SSBR and NHEJ, respectively (Koch et al., 2004; Loizou et al., 2004). Interaction of non-phosphorylated XRCC1 and XRCC4 with PNKP also stimulates PNKP catalytic activity although the binding is to the catalytic domain rather than the FHA domain (Lu et al., 2010; Mani et al., 2007; Whitehouse et al., 2001).

The *PNKP* mutations identified in the MSCZ patients to date appear mostly in the phosphatase and kinase domains (Fig. 2) (Nakashima et al., 2014; Shen et al., 2010). The patient with the 163G > T (p.A55S) mutation in the FHA domain also carries a 874G > A (p.G292R) mutation in the phosphatase domain (Nakashima et al., 2014). The mutations responsible for AOA4, in contrast, all appear in the kinase domain (Bras et al., 2015; Paucar et al., 2016; Tzoulis et al., 2016). Intriguingly, the mutation giving rise to the T424G-frameshift was found in four of the MCSZ families and in an AOA4 proband and in the brothers exhibiting the combined symptoms of MSCZ and AOA4 (Bras et al., 2015; Poulton et al., 2013; Shen et al., 2010). In addition, a homozygous recessive missense mutation in the FHA domain of *PNKP* (58G > A, p.P20S) was identified in a proband with epileptic encephalopathy but with no sign of microcephaly or developmental delay (Carvill et al., 2013).

4.2. Mitochondrial PNKP

PNKP localizes to mitochondria as well as the nucleus (Mandal et al., 2012; Tahbaz et al., 2012). The mitochondrial PNKP appears to be the same full-length isoform as the nuclear protein (Tahbaz et al., 2012) and thus any mutations in *PNKP* will affect the mitochondrial protein as well as the nuclear protein. In mitochondria PNKP binds to NEIL2 and to DNA polymerase γ , indicating a role for PNKP in mitochondrial BER (Mandal et al., 2012). Depletion of cellular PNKP increased the levels of SSBs in untreated as well as hydrogen peroxide-treated cells (Mandal et al., 2012; Tahbaz et al., 2012). Both the kinase and phosphatase activities were required to fully restore the DNA in the hydrogen peroxide-treated cells (Tahbaz et al., 2012).

4.3. MCSZ cells and PNKP knockout mice

Mutations in PNKP that give rise to neurological disorders are shown in Fig. 2. An examination of Epstein-Barr virus-transformed lymphocytes derived from affected MCSZ individuals indicated a substantial decrease in their PNKP protein content, and their repair of damage induced by either hydrogen peroxide or camptothecin was severely impaired (Reynolds et al., 2012; Shen et al., 2010). Analysis of the kinase activity of the mutated proteins revealed that the frameshift mutations in the kinase domain abrogated kinase activity, while the E326K protein had similar activity as the wild-type protein (Reynolds et al., 2012). Interestingly, the L176K protein showed reduced kinase activity despite being located in the phosphatase domain. A similar analysis revealed that only the L176K mutation resulted in a partial decrease in phosphatase activity (Reynolds et al., 2012). Skin fibroblasts isolated from the two affected brothers exhibiting the combined symptoms of MCSZ and AOA4 also displayed reduced PNKP protein content and increased apoptosis in response to stress induced by serum starvation followed by treatment with dithiothreitol (Poulton et al., 2013).

Unlike *Tdp1* and *Aptx*, embryo-wide knockout of *Pnkp* is embryonic lethal in mice, as is homozygous introduction of the murine T424G-frameshift mutation (Shimada et al., 2015). However, Shimada et al. were able to generate a Nestin-cre knockout just targeting the nervous system that died 5 days after birth, and more importantly, a viable hypomorphic conditional knockout with a smaller brain that more closely mimics MCSZ (Shimada et al., 2015). Analysis of neurogenesis in these mice revealed increased DNA damage and apoptosis in the neocortex of E13.5 embryos and a reduction in proliferating cells. Apoptosis was shown to be dependent on p53. A less overt, but nonetheless discernable, response was observed when the loss of PNKP commenced after birth. For example, there was a significant reduction in the level of myelin basic protein required for myelination of oligodendrocytes.

4.4. Mechanism underlying MCSZ and AOA4

Several mutations in *PNKP* give rise to MCSZ and AOA4. Those responsible for MCSZ lead to a decrease in the cellular levels of PNKP, down to 5–10% of wild-type levels in EBV-transformed lymphocytes (Reynolds et al., 2012; Shen et al., 2010). The reduction is probably not due to lower transcriptional levels (Shen et al., 2010), but more likely due to protein instability (Reynolds et al., 2012). At this point it is understandable that the proteins

produced by the frameshift mutations would show reduced stability, but it is less clear why the L176F and E326K modified proteins should be less stable than the wild-type protein. In addition, the L176F modified protein showed reduced phosphatase activity (Reynolds et al., 2012). Leu176 is packed in the hydrophobic core of the phosphatase domain, and positioned immediately under the phosphatase active site cleft. Mutation to the slightly larger phenylalanine residue could introduce steric clashes that may distort the phosphatase active site geometry (Reynolds et al., 2012). The E326K mutation does not appear to interfere with the catalytic activity of recombinant PNKP and yet the severity of the resultant MCSZ is similar to that arising from the other mutations. This may be due to an intrinsic loss of stability, but we speculate that it is also possible that in the wild-type protein the Glu326, which is exposed on the surface of the phosphatase domain distant from the active site and DNA binding surface (Bernstein et al., 2005), may interact with XRCC4-Lig4 in a way that modulates PNKP function. The fact that the E326K MCSZ mutation is a charge swap is consistent with an electrostatic interaction between this region and a partner in XRCC4-Lig4. It is instructive that none of the mutations in either MSCZ or AOA4 abolish the PNKP phosphatase activity, perhaps indicating that complete loss of this function would be lethal during neurogenesis as a result of the frequency of generating 3'-phosphate termini and lack of an efficient backup pathway.

The murine *Pnkp* knockout model clearly established that loss of Pnkp is considerably more detrimental than loss of either Tdp1 or Apx and this may explain why mutations in *PNKP* give rise to a severe neurodevelopmental disorder as well as a neurodegenerative disorder. Mechanistically, this may be due to the important role PNKP plays in both SSBR, the loss of which may underlie AOA4, and NHEJ, which if partially abrogated may be the cause of MCSZ. In a similar vein, Shen et al. suggested that the symptoms of MCSZ itself reflected PNKP's participation in both repair pathways by pointing out that mutations to the NHEJ proteins LIG4 and XRCC4 also cause microcephaly while mutations to the SSBR protein XRCC1 causes seizure-like behavior (Shen et al., 2010). However, it is hard to reconcile these ideas with the perplexing T424G-frameshift mutation common to both MCSZ and AOA4 patients. It implies that factors beyond the site of mutation in *PNKP* contribute to the disease process. For example, it has recently been shown that exposure of cells to cholesterol, which is abundant in the brain, stimulates PNKP expression in a human lymphoblastic lymphoma cell line (Codini et al., 2016).

The hypomorphic *Pnkp* model was able to partially recapitulate the symptoms of MCSZ (Shimada et al., 2015). Importantly, it revealed that the reduction of functional PNKP during neural development led to increased DNA damage and p53-dependent apoptosis in neural progenitor cells. Deleting PNKP postnatally also caused progressive damage to post-mitotic neurons, although it was not clear to what extent this mimics AOA4. Nonetheless, the investigators suggested that the loss of oligodendrocytes might be due to disruption of transcription in the DNA damaged cells.

5. Conclusions

Neurological problems are a common consequence of mutations in genes encoding DNA repair proteins in several DNA repair pathways. Here we have discussed the

neurodegenerative and neurodevelopmental diseases arising from mutations in genes coding for proteins that process DNA strand-break termini. Many outstanding issues remain before we obtain a complete picture of the link between DNA damage and neurodegeneration and neurodevelopment. From the perspective of molecular mechanism(s), we still need to clarify the respective roles of single and double-strand break repair and the relative importance of damage to nuclear vs mitochondrial DNA. Most attention has focused on nuclear DNA, but it is clear that mitochondria pathology contributes significantly to neurodegenerative diseases including Parkinson's disease (Ryan et al., 2015) and disorders involving epilepsy (Zsurka and Kunz, 2015). We then need to understand the consequences of unrepaired damage – are the downstream consequences related to one or a specific set of genes or proteins or are the responses driven by a global effect such as inhibition of transcription? Next we have to consider the subset of cells that appear to be affected by each of the disorders discussed. This will require new tools such as murine cerebellar organotypic cultures (Tzur-Gilat et al., 2013), and alternative animal models, especially for SCAN1, AOA1 and AOA4.

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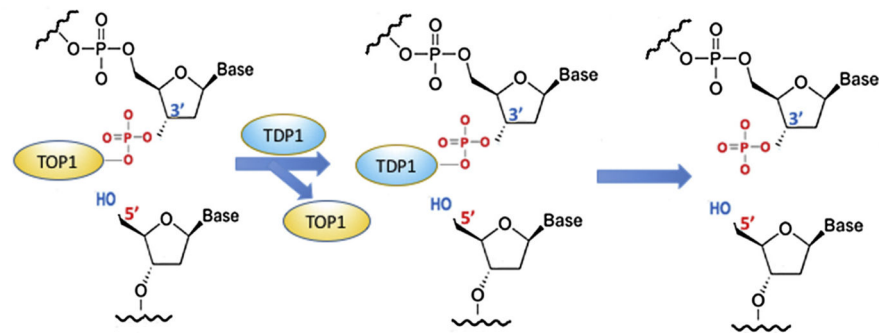
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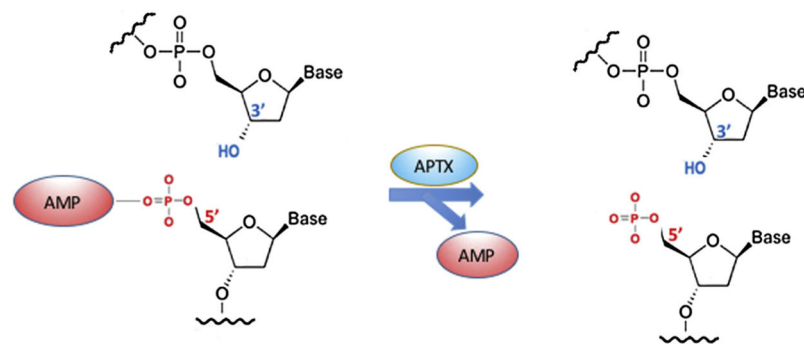
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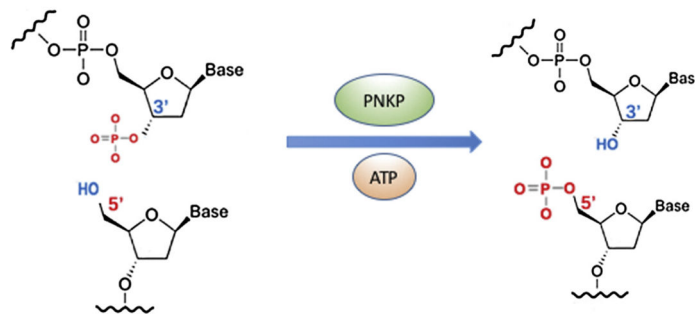
A. TDP1



B. APTX



C. PNKP

**Fig. 1.**

Reactions catalyzed by DNA strand temini-processing enzymes. (A) TDP1 acts on stalled TOP1 cleavage complexes. Following partial proteolysis of TOP1 covalently bound to the 3'-phosphate, TDP1 carries out nucleophilic attack of the TOP1-DNA phosphotyrosyl bond to form a transient covalent TDP1-DNA intermediate, which subsequently undergoes hydrolysis catalyzed by TDP1 itself. (B) APTX catalyzes the removal of 5'-AMP from abortive ligation intermediates. (C) PNKP catalyzes the phosphorylation of 5'-OH termini, using ATP as the phosphate donor, and the dephosphorylation of 3'-phosphate termini.

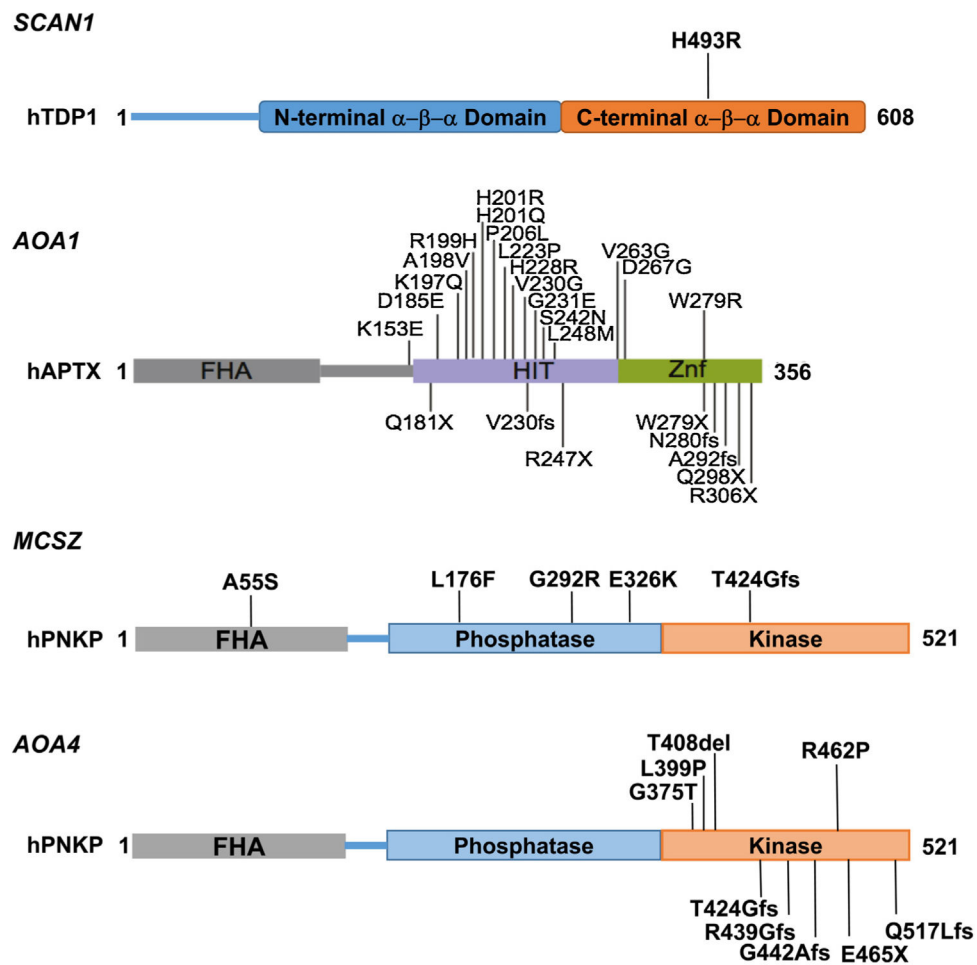


Fig. 2. Protein alterations as a result of mutations in *TDPI*, *APT X* and *PNKP* responsible for SCAN1, AOA1, MCSZ and AOA4. FHA represents the forkhead associated domains in *APT X* and *PNKP*, HIT and Znf represent the histidine triad and Zinc finger domains in *APT X*. Frameshifts are indicated by fs, nonsense mutations by an X and deletions by del. The numbers on the righthand side of each protein indicate the size of each protein in terms of the number of amino acid residues. Not shown in the MSCZ figure is a deletion of exon15 accompanied by a premature stop codon arising from a mutation in intron 15 that disrupts normal splicing. (Adapted from Schellenberg et al., 2015 and Bras et al., 2015).