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## Tyrosyl-DNA Phosphodiesterase I a critical survival factor for neuronal development and homeostasis

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### Abstract

Tyrosyl-DNA phosphodiesterase I (TDP1), like most DNA repair associated proteins, is not essential for cell viability. However, dysfunctioning TDP1 or ATM (ataxia telangiectasia mutated) results in autosomal recessive neuropathology with similar phenotypes, including cerebellar atrophy. Dual inactivation of TDP1 and ATM causes synthetic lethality. A TDP1H<sup>493</sup>R catalytic mutant is associated with spinocerebellar ataxia with axonal neuropathy (SCAN1), and stabilizes the TDP1 catalytic obligatory enzyme-DNA covalent complex. The ATM kinase activates proteins early on in response to DNA damage. Tdp1<sup>-/-</sup> and Atm<sup>-/-</sup> mice exhibit accumulation of DNA topoisomerase I-DNA covalent complexes (TOPO1-cc) explicitly in neuronal tissue during development. TDP1 resolves 3'- and 5'-DNA adducts including trapped TOPO1-cc and TOPO1 protease resistant peptide-DNA complex. ATM appears to regulate the response to TOPO1-cc via a noncanonical function by regulating SUMO/ubiquitin-mediated TOPO1 degradation. In conclusion, TDP1 and ATM are critical factors for neuronal cell viability via two independent but cooperative pathways.

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Tyrosyl-DNA phosphodiesterase I (TDP1), a eukaryotic DNA repair enzyme that belongs to the phospholipase D super family<sup>1-3</sup>, is ubiquitously expressed in most if not all human and mouse tissue, from neurons to peripheral skeletal cells<sup>4</sup>. In the cell, TDP1 is detected in the nuclear-, cytosolic- and mitochondrial-compartments<sup>4,5</sup>. TDP1 is able to resolve a wide variety of phospho-adducts from the 3' and 5' ends of nicked DNA strands. Tdp1 substrates vary from small adducts, such as oxidative DNA damage and chain terminating nucleotides, to large adducts including potentially lethal protein-DNA covalent complexes or the protease-resistant peptides that are still covalently linked to the DNA after degradation<sup>6,7</sup>. Protein-DNA adducts include DNA topoisomerases (TOPOs) covalently linked to the DNA via a 3'phospho-tyrosyl or 5'phospho-tyrosyl linkage, representing a TOPO1-DNA (TOPO1-cc) or TOPO2-/TOPO3-DNA covalent complex, respectively<sup>8-10</sup>. In addition, TDP1 is able to hydrolyze a 3'phospho-histidyl linkage or TDP1 covalently bound to the DNA (TDP1-cc)<sup>11-13</sup>. Note: higher eukaryotic cells contain an additional enzyme called TDP2/TTRAP that resolves the 5'phospho-tyrosyl linkages more efficiently than TDP1<sup>14-17</sup>.

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However, this enzyme is absent in yeast cells in which TDP1 resolved both 3' and 5' phospho-tyrosyl linkages<sup>18</sup>.

To resolve this eclectic array of phospho-adducts, TDP1 utilizes the coordinated action of two catalytic histidines (Figure 1). In the case of hydrolyzing a 3' phospho-tyrosyl linkage, the N-terminally located histidine functions as a nucleophile (His<sup>nuc</sup>; His<sup>263</sup> in human (h)Tdp1 and His<sup>182</sup> in yeast (y)Tdp1) to attack the 3' phospho-tyrosyl linkage to form a 3' phospho-histidyl bond. The C-terminally located histidine acts as a general acid/base (His<sup>gab</sup>; His<sup>493</sup> in hTdp1 and His<sup>432</sup> in yTdp1) that activates a water molecule to hydrolyze the Tdp1-DNA linkage formed in the first step, resulting in separation of Tdp1 from the DNA<sup>1,2,11,19</sup>.

A substitution of the hTdp1His<sup>gab</sup> to Arginine (hTdp1H<sup>493</sup>R) has been identified as the molecular basis for the rare autosomal recessive neurodegenerative disease Spinocerebellar Ataxia with Axonal Neuropathy or SCAN1<sup>13</sup>. SCAN1 symptoms are similar to Ataxia Telangiectasia (A-T) and Ataxia with Oculomotor Apraxia (AOA1). A-T results from defects in the serine/threonine protein kinase, DNA damage response regulator ATM (ataxia telangiectasia mutated<sup>20</sup>, and AOA1 is caused by a defect in the DNA repair enzyme aprataxin (APTX)<sup>21</sup>, respectively<sup>13,22</sup>. ATM is activated in response to the detection of double-strand breaks via the Mre11-Rad50-Nsb1 (MRN) complex to phosphorylate a plethora of downstream proteins, including H<sub>2</sub>AX and p53<sup>23,24</sup>. On the other hand, APTX is an adenylyl-hydrolase that resolves 5' adenylylated-DNA (5' AMP-DNA) adducts as a result of abortive DNA ligase activity<sup>21,25</sup>. The importance of DNA "end-processing" enzyme activities became even more evident with the observation that mutations in the kinase domain of polynucleotide kinase phosphatase (PNKP) are associated with AOA4, while other substitution in PNKP are detected in patients with microcephaly with seizures (MCSZ) (26-28). Moreover, loss of TDP2 activity is associated with autosomal recessive spinocerebellar ataxia-23 or SCAR-23<sup>29,30</sup>.

SCAN1 patients demonstrate a progressive cerebellar atrophy that results in ataxia symptoms during late childhood (13-15 years). Intriguingly, this atrophy only seems to affect the cerebellar neurons within the vermis region of the cerebellum<sup>13,22</sup>. Moreover, these patients do not show an increase in cancer predisposition, immunodeficiencies, or cardiomyopathy. Takashima and colleagues originally proposed that the H<sup>493</sup>R substitution would inactivate the enzyme<sup>13</sup>; however, subsequent biochemical studies revealed a decreased dissociation rate resulting in an increased level of TDP1<sup>SCAN1-cc</sup> (Figure 1)<sup>31-33</sup>. Moreover, the only available resolved crystal structure of a Tdp1His<sup>gab</sup>Arg mutant enzyme is the yeast analogous substitution (yTdp1H<sup>432</sup>R)<sup>32</sup>. The yTdp1H<sup>432</sup>R crystal structure demonstrated that the arginine side chain reduces the depth of the TDP1 catalytic pocket, potentially obstructing a water molecule to enter the pocket at the correct position<sup>32</sup>. On the other hand, arginine is also a weaker general acid/base than histidine, which affects the activation rate of the water molecule that facilitates dissociation of Tdp1 from the DNA. Additionally, the arginine guanidinium moiety changes the electrostatic charge distribution within the catalytic pocket to highly positive<sup>32</sup>. All these factors contribute to the reduced dissociation rate of TDP1His<sup>gab</sup>Arg from the DNA. The biochemical/biophysical characteristics of the His<sup>gab</sup>Arg substitution as well as the mild cellular toxicity induced by

expression of this TDP1H<sup>gab</sup>R-mutant are conserved between yeast and human TDP1<sup>11,31-33</sup>. Moreover, examination of additional substitutions of the His<sup>gab/432</sup> in yTdp1 showed that a His<sup>432</sup>Lys substitution resulted in a minor (~10-fold) decrease in catalytic activity with no detectable toxicity<sup>32</sup>. Conversely, expression of His<sup>432</sup> substitution with residues that contain a smaller polar or aliphatic side chain such as Asn, Glu, Ser, Thr, Leu, Val and Ala, displays an acute toxicity, albeit recessive to wild type Tdp1. These observations also revealed that the SCAN1 mutation (His<sup>gab</sup>Arg) among the toxic His<sup>gab</sup> mutants exhibits only a mildly toxic phenotype. Similar to the SCAN1 mutant, the toxicity induced by expression of the His<sup>gab</sup>Asn mutant is correlated with increased cellular levels of enzyme-DNA intermediates and a reduction in catalytic activity in both yeast and human TDP1<sup>11,31-34</sup> [Cuya, van Waardenburg manuscript under revision Oncotarget]. It is therefore intriguing that more TDP1 single nucleotide polymorphisms are not identified and associated with neuronal syndromes or other diseases associated with genome instability etiology. Although substitution of either catalytic histidine results in a gain of function (toxicity and reduced activity)<sup>11,32,34</sup>, two questions remain open: How does the Tdp1H<sup>493</sup>R mutant enzyme cause the SCAN1 pathology? and Why are (cerebellar) neurons so sensitive to this toxic Tdp1 mutant?

Tdp1, like most DNA repair proteins/enzymes, is not essential for eukaryotic cell viability. This is generally due to the existence of redundant DNA repair processes that are able to resolve protein-DNA adducts. However, some human carcinomas developed a dependency on/or addiction of Tdp1 expression<sup>35,36</sup>. Currently, no mouse model for SCAN1 that expresses the His<sup>gab</sup>Arg substitution has been generated. However, three different groups generated a Tdp1 knockout mouse<sup>37-39</sup>. Interestingly, Tdp1<sup>-/-</sup> mice do not develop any ataxia or neuropathy symptoms related with SCAN1 or other behavioral phenotypes, and their electrophysiology is comparable to their wild-type<sup>37-39</sup>. However, these Tdp1<sup>-/-</sup> mice do develop age-dependent progressive cerebellar atrophy, and display one of the non-neuronal related SCAN1 symptoms, hypoalbuminemia<sup>13,22,39</sup>. The Tdp1<sup>-/-</sup> mice also exhibit an expected hypersensitivity to camptothecin (CPT) or topotecan treatment (a FDA approved camptothecin chemotherapeutic) that was evident in proliferating intestinal cells and hematopoietic cells<sup>37-39</sup>. Moreover, Tdp1<sup>-/-</sup> fibroblast extracts show a deficiency in their ability to process 3'phospho-glycolate adducts within double-stranded DNA breaks but not single-strand breaks, and these cells show an increased bleomycin sensitivity<sup>37,38</sup>.

On the other hand, Mckinnon and coworkers reported that TDP1 and ATM are critical during the development of neuronal cells to control TOPO1 induced DNA damage (40). Mice can survive the individual inactivation of ATM or TDP1; however, dual inactivation causes synthetic lethality. The *Atm*<sup>-/-</sup>,*Tdp1*<sup>-/-</sup> combination showed to be embryonically lethal between E13.5 and E16.5, a period in which the TOPO1-cc levels are at their maximum<sup>40</sup>. TOPO1 can be trapped onto the DNA by endogenous DNA lesions, such as abasic sites and single-strand nicks which can occur due to oxidative damage<sup>41,42</sup>. Indeed, *Tdp1*<sup>-/-</sup> and *Atm*<sup>-/-</sup> mice show elevated levels of TOPO1-cc in cortical/cerebella tissue during embryonic development up to one year after birth, while control cells or other tissue in the knockout mice show no or minor levels of TOPO1-cc<sup>40</sup>. This suggests that during development TOPO1-cc lesions accumulated specifically in the neuronal tissue and that for a normal homeostasis ATM and TDP1 regulation of processing stalled TOPO1-cc is critical.

The accumulation of TOPO1-cc and single-strand DNA breaks in the ATM<sup>-/-</sup> and Tdp1<sup>-/-</sup> quiescent primary astrocytes is even more pronounced after camptothecin (CPT), ionizing radiation, or hydrogen peroxide treatment. ATM's response to TOPO1-cc in neuronal cells is independent of its kinase activity or other canonical functions. Inactivation of MRE11, DNA-PKcs, or LIG4 in combination with Tdp1<sup>-/-</sup> exhibits a normal Mendelian offspring distribution without an obvious phenotype<sup>40</sup>. However, inactivation of XRCC1 [the scaffold protein within the base excision repair (BER) pathway, which partners include TDP1<sup>43</sup>] showed elevated levels of TOPO1-cc similar to Atm inactivation that are lower than Tdp1 inactivation. Inactivation of Xrcc1 did not reduce the protein levels of TDP1 or other BER affiliated proteins, while the combination of Atm and Xrcc1 inactivation accumulates TOPO1-cc levels similar to Tdp1 inactivation. In an effort to identify the non-canonical function of ATM, Katyal et al observed that ATM is able to regulate post-translational modification (PTM) of TOPO1 by SUMOylation of TOPO1 and ubiquitination via a yet unknown pathway<sup>40</sup>. These ATM stimulated PTMs in response to CPT treatment, stimulate proteasome-mediated degradation of TOPO1 which was prevented by proteasome inhibitor MG132. The response was lost in A-T cells but still active after treatment with an ATM inhibitor, suggesting that ATM kinase activity is not involved in this response.

In conclusion, both ATM and TDP1 play a critical protective role during neuronal development to prevent accumulation of TOPO1-cc (Figure 2). ATM neuronal cells via a novel non-canonical activity, which does not require ATM kinase function. ATM regulates posttranscriptional modification of TOPO1 and potentially TOPO1-cc by stimulating SUMOylation, followed by ubiquitination that promotes proteasome-mediated TOPO1 degradation (Figure 2). Although no SUMO E3-ligases or ubiquitin E2-conjugation/E3-ligase complexes have been identified, in yeast two potential complexes were identified. First is the SUMO ligase Pli1 mediates SUMO modification of TOPO1 while the SUMO-targeted ubiquitin ligase Slx8 was shown to mediate ubiquitin modification<sup>44</sup>. The second complex is the DNA-, SUMO- and ubiquitin-dependent metalloprotease complex WSS1/CDC48/DOA1 might be responsible for the degradation of modified TOPO1-cc<sup>45</sup>. The WSS1 human analog is Spartan/DVC1 protein. TDP1, on the other hand, hydrolyzes the 3'phospho-tyrosyl linkage that covalently attaches full length TOPO1 with the 3'phosphoryl end of a DNA strand or the protease resistant TOPO1-peptide<sup>1,11</sup>. Thus, TDP1 plays a critical role in removal of a protein/peptide-DNA adduct during neuronal development (Figure 2). These studies suggest a potential molecular basis for the etiology of SCAN1; not only TOPO1-cc are accumulating during embryonic development but they are replaced by TDP1H<sup>493</sup>R-cc, which together maximizes the ability of ATM and the other repair pathways to maintain cell viability, resulting in a slow but progressive cerebellar atrophy (Figure 2). Moreover, the work of McKinnon and coworkers<sup>40</sup> gave the first clue, specific accumulation of TOPO1-cc in the neuronal cells, of why the cerebellar is specifically affected and not other tissues.

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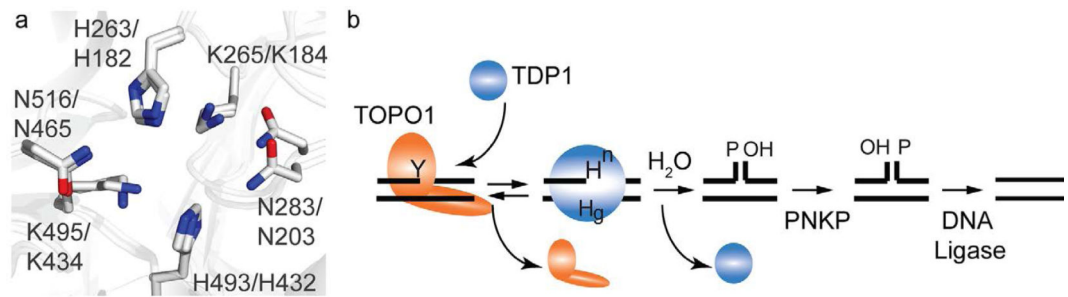
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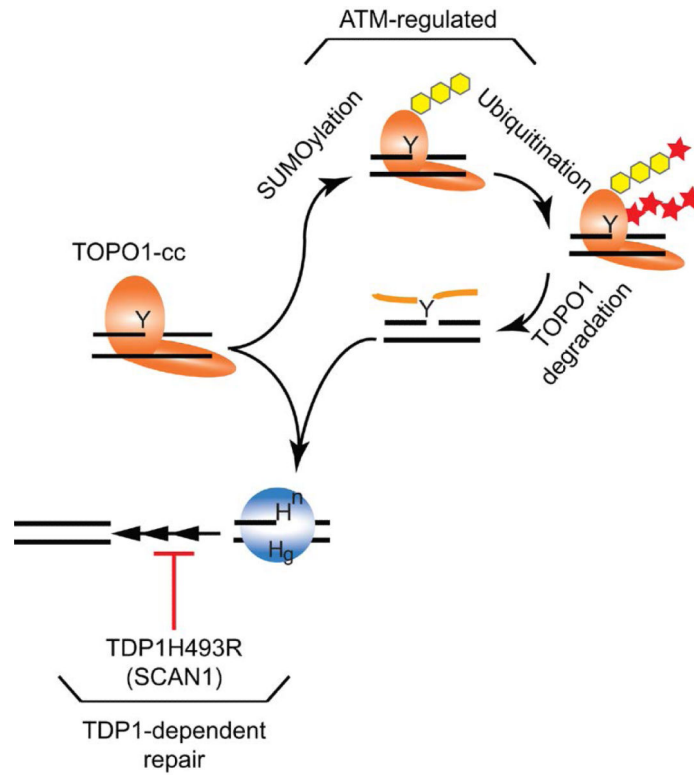
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**Figure 1.**

The Tdp1 catalytic pocket and mechanism is conserved from yeast to human. (a) Overlay of the crystal structure catalytic pockets of yeast and human Tdp1 showing the two catalytic HxKx<sup>n</sup>N-motifs; H263, K265, N283 N-terminal motif and H493, K495, N516 C-terminal motif in human TDP1. H182, K185, N203 N-terminal motif and H432, K435, N434 C-terminal motif in yeast TDP1. Residues H263 in human TDP1 and H182 of yeast TDP1 represent the nucleophilic histidine (His<sup>nuc</sup>), while H493 of human TDP1 and H432 in yeast TDP1 represent the general acid/base histidine (His<sup>gab</sup>). [human TDP1; PDB # 1NOP<sup>46</sup> and yeast TDP1; PDB# 1Q32<sup>11</sup>]. (b) Tdp1 catalytic of DNA topoisomerase I-DNA covalent complexes (TOPO1-cc); Tdp1 resolves the 3'-phosphotyrosyl linkage (Y) via nucleophilic attack of the His<sup>nuc</sup> (H<sup>n</sup>), that releases TOPO1. This step generates the obligatory TDP1-DNA intermediate via 3'-phospho-amide linkage. Water is activated by His<sup>gab</sup> (H<sub>g</sub>) to hydrolyze the TDP1-DNA linkage allowing TDP1 to dissociate from the DNA. The exciting single-strand nick requires further processing by polynucleotide kinase-phosphatase (PNKP) prior to DNA ligation.





**Figure 2.**

TOPO1-cc processing by TDP1 and ATM independent pathways in neuronal cells. The removal of accumulated TOPO1-cc in neuronal cells during development by noncanonical ATM-regulated SUMOylated (yellow hexagon) followed by ubiquitination (red star) of TOPO1 that results in TOPO1 degradation potentially proteasome-mediated<sup>40</sup> or by SUMO stimulated selective ESCRT vesicle-trafficking mediated autophagy<sup>47</sup>. The protease-resistant covalently linked TOPO1-peptide and, independent of ATM, TOPO1-cc can be directly removed by TDP1-mediated hydrolysis. In SCAN1 patients, the TDP1H<sup>493</sup>R mutant enzyme will be able to hydrolyze both TOPO1-cc and the TOPO1-peptide fragment from the DNA but will form a stable TDP1H<sup>493</sup>R-cc<sup>11,13,31-33</sup>. The accumulated TDP1H<sup>493</sup>R-cc together with the TOPO1-cc or TOPO1-peptide-DNA complex will maximally challenge the ATM and other DNA repair pathways, resulting in slow progressive cerebellar atrophy.