Putative Role of the Futile Repair Initiated by Human Thymine-DNA Glycosylase in Formation of Programmed Strand Breaks in Neuronal Enhancers

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Abstract. Enhancers are regulatory DNA elements that play a crucial role in controlling gene expression in specific cell types, including neurons. Enhancer activity is tightly regulated and involves the recruitment of various proteins and enzymes to facilitate the opening of chromatin and the activation of target genes. Given the importance of enhancers in neuronal function, the presence of single-strand DNA breaks (SSBs) in these regions raises intriguing questions about their potential impact on gene regulation and neuronal activity. Single-strand DNA breaks (SSBs) have been identified as important lesions in the genome, with the potential to influence gene expression and genomic stability. By understanding the role of SSB repair and human mono-functional Thymine-DNA glycosylase (TDG) catalyzed futile excision of regular bases in enhancer regions, we may gain insights into the molecular mechanisms underlying neurological disorders and potentially identify new therapeutic targets for intervention.

1 Introduction

Enhancers are non-coding segments in the genome that trigger the expression of specific genes transcribed by RNA polymerase II (RNAPII). They can function independently of orientation, distance, and relative position in relation to the target gene [1], with the potential to be located up to a million base pairs away from the gene, as demonstrated by the SHH gene [2]. In vertebrates, enhancers typically range from 100 to 1000 bp in length, and they can form clusters to create super-enhancers [3]. They are predominantly located in intergenic and intronic regions, though some enhancers have been observed within exons. Enhancers are comprised of dense collections of binding sites for transcription factor (TFBS), which serves for binding by specific TFs for different cell types, coregulators, chromatin modifiers, architectural proteins such as cohesin, condensin, CTCF, other enzymes, and RNAPII. Due to the extensive assembly of proteins, enhancers are often depleted of nucleosomes and thus

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susceptible to nucleases, which reflects DNA accessibility – a characteristic frequently used as a hallmark of enhancers [4]. Once assembled, the enhancer complex loops over and physically contacts the target promoter to activate transcription. In recent studies single-strand DNA breaks (SSBs) were shown to be enriched in enhancer regions of neurons [5]. Single-strand break repair (SSBR) is a crucial process for maintaining the integrity of DNA in enhancer regions, which play a critical role in gene regulation. These enhancer regions are prone to DNA damage and are characterized by SSBR hotspots, where excision and de novo DNA synthesis are highly active. The SSBR hotspots in enhancer regions involves coordinated interactions between various repair pathways, including base excision repair (BER) and nucleotide excision repair (NER), to restore DNA integrity effectively and precisely. Understanding the specific SSBR hotspots in enhancer regions is essential for gaining insights into the impact of DNA damage on gene expression regulation and the maintenance of genomic stability [6-7]. Furthermore, it is important to investigate the factors that influence the SSBR hotspots in enhancer regions to elucidate their role in preserving the functionality of these critical genomic elements. One enzyme that has emerged as a key player in the SSBR is human thymine DNA glycosylase (TDG) [8]. TDG is a DNA repair enzyme that specifically recognizes and excises thymine, uracil mispaired with guanine in CpG context [9]. This enzyme helps to maintain the integrity of the genome and prevent mutations that can lead to diseases such as cancer [10]. In addition to its role in base excision repair, TDG has been involved in the regulation of gene expression through its involvement in the active demethylation of DNA removing base generated by oxidative conversion of 5-methylcytosine into 5-formylcytosine and 5-carboxylcytosine [11-12]. It was demonstrated that under certain circumstances TDG enzyme acts in error-prone aberrant manner that refers to the potential of this enzyme to excise the regular T residues opposite to A* (damaged adenine) in double-stranded DNA, which can lead to mutations and genomic instability [13]. While TDG normally recognizes and removes mismatched bases (T, U) that are inappropriately incorporated into DNA (G•T, U•G), aberrant repair can occur when it targets and removes regular thymine mismatched with damaged adenine, such as Hypoxanthine. This process has the potential to introduce mutations and disrupt the control of gene expression via epigenetics [13].

Our unpublished data have suggested a potential involvement of TDG in the formation of SSBs within enhancers presents an intriguing avenue for understanding the intricate interplay between DNA repair and gene regulation in non-dividing cells. We have demonstrated that hTDG performs excision of T and C in regular non-damaged DNA, under physiological conditions, through futile DNA glycosylase activity. Moreover, TDG-mediated DNA demethylation may also contribute to the dynamic regulation of enhancer activity in neurons [14]. In light of these recent findings, the present work aims to explore the potential involvement of TDG in formation of SSBs in enhancer regions of neurons and the possible molecular mechanism of this process. By elucidating the mechanisms through which SSBs and TDG may impact enhancer function in neurons, this research could provide valuable insights into the interplay between DNA damage, DNA repair, and gene regulation in the nervous system.

2 Materials and Methods

2.1 Enzyme expression and purification

The plasmid containing full-length hTDG (pET28c-hTDG) was transformed into bacterial cells (Arctic Express (DE3)), acquired from Novagen-EMD4 Biosciences (Merck Chemicals, Nottingham, UK), by using electroporation and subsequently sorted on Luria Broth (LB)

plates in the presence of kanamycin at a concentration of 50 μg•ml^{-1}, due to the fact that the expression vector pET28c contains an antibiotic resistance gene. Further the cells bearing the plasmid were grown at 37° C in a liquid LB medium in a larger volume up to the indicator of OD_{600nm} = 0.6-0.8, to obtain biomass, to which 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was later added to initiate protein induction. After overnight induction at 12°C, the cells were pelleted and solubilized in Resuspension buffer (20 mM HEPES-KOH pH 7.6, 40 mM NaCl) supplemented with CompleteTM Protease Inhibitor Cocktail (Roche Diagnostics, Switzerland) and subsequently lysed using French press at 18,000 psi. Following actions were carried out at 4°C. The supernatant obtained after centrifugation for 2h at 12000 x g of cell lysate was adjusted to buffer A containing 20 mM HEPES, 500 mM NaCl, 20 mM imidazole for further purification on a HiTrap Chelating HP column (Amersham Biosciences, GE Health) using affinity-chromatography. His-tagged proteins were then eluted with imidazole gradient (20-500 mM). Due to the presence of impurities, additional purification was carried out using a column HiTrap Heparin HP, from which in turn protein was eluted with gradient of high salt concentrations. The resulting fractions of purified enzyme were tested on a SDS-PAGE. Fractions with high concentration of purified protein that were determined by the method of Bradford [15] and stored at -80°C in 50% glycerol to maintain enzyme activity for further analysis.

2.2 Oligonucleotides labeling and hybridization

The nucleotide sequence of synthetic oligonucleotides shown in Table 1 was designed according to the sources, considering the preferred contexts of the dinucleotide, and obtained from Eurogentec (Seraing, Belgium). The single-stranded oligonucleotide labelling reaction was carried out in PNK buffer A containing 500 mM Tris-HCl (pH 7.6 at 25 °C), 100 mM MgCl2,50 mM DTT, 1 mM spermidine in the presence of 3,3 pmol γ-^{32}P and Polynucleotide kinase T4 (PNK T4) from Thermo Scientific™ (Waltham, Massachusetts) that catalyzes the transfer of gamma phosphate from ATP to the 5'-OH group of the DNA chain. Hybridization was performed in annealing buffer (200mM HEPES pH 7.6, 0.5M KCl) and in the presence of labelled oligonucleotides and their complementary strands at 80°C followed by gradual cooling.

Table 1. DNA sequence of examined oligonucleotides

Name of oligonucleotides	DNA sequence of oligonucleotide substrates	Length	References
SMYD3-Enh	ATCTCCTGACCTCATGATCCGCCCGCCTC	29 mer	[16]
Enh-c.SMYD3	GAGGCGGGCGGATCATGAGGTCAGGAGAT	29 mer	[16]
C>T-ADNP	AAAAGGCAGATATCCCTGTAAAAAGTTCAC	30 mer	[17]
c.C>T-ADNP	GTGAACTTTTTACAGGGATATCTGCCTTTT	30 mer	[17]
ADNP-T>C	AAAAGGCAGATATCCCCGTAAAAAGTTCAC	30 mer	[17]
c.ADNP-T>C	GTGAACTTTTTACGGGGATATCTGCCTTTT	30 mer	[17]
CD55-Alzh	CTTTGAGCTCCAAAAGCACACTGGTTACAC TGTCAACTTTA	51 mer	[18]
Alzh-c.C D55	TAAAGTTGACAGTGTAACCAGTGTGCTTTT GGAGCTCAAAG	51 mer	[18]
Lhx2_c-MYC	TTCAAAGACTGCCACGTGCCTTAGGAATAC	30 mer	[19]
c-MYC_c.Lhx2	GTATTCCTAAGGCACGTGGCAGTCTTTGAA	30 mer	[19]
AUST2-CTCF	TGAACTCTGGCCACCAGGGGGCGCTCTGCA	30 mer	[20]
CTCF-c.AUST2	TGCAGAGCGCCCCCTGGTGGCCAGAGTTCA	30 mer	[20]

2.3 DNA glycosylase activity assay

To study futile activity of hTDG on regular double strand DNA, 20 nM of labeled DNA duplexes and 200 nM of purified recombinant protein were incubated in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 μg•ml-1 BSA at 37°C for 1 h and 18 h. For measuring specific activity of hTDG 5'-[32P]-labelled oligonucleotide duplex containing T•G mismatch was incubated with enzyme for 1 h at 37°C to use it as a control. After indicated incubation time 0,1 M NaOH was added to reaction mixture and heated for 3 min at 95°C to obtain cleavage of abasic sites left after glycosylase activity of TDG. Subsequently samples were neutralized with 0,1 HCl. To determine the size of the resulting reaction product, markers were obtained using nucleases: Endonuclease IV (Nfo1) purified as described [21] and Exonuclease III (XthA) from New England Biolabs (Evry, France). For each DNA duplex corresponding 5'-[32P]-labelled oligonucleotide in concentration of 20nM was in incubated for 1h at 37°C in the presence of 1μM of Endonuclease IV (Nfo1) in the suitable buffer (25mM HEPES KOH pH 8.4, 100 μg•ml-1 BSA) and for 10 minutes at room temperature (RT) with 1u of Exonuclease III (XthA) in NEB1 buffer (10 mM Bis-Tris-Propane-HCl, 10 mM MgCl2, 1 mM DTT, pH 7). The reaction was stopped by adding stop solution (0.1 M NaOH, 10mM EDTA, 0.25 % SDS) and heating up to 95°C for 5 min. Before loading to denaturing polyacrylamide gel of 20% (w/v) to get rid of salts reaction mixtures were passed through the column equilibrated in 7.5 M urea (Sephadex G25 column, Amersham Biosciences) and separated at 42°C in 0,5X TBE buffer. Before scanning at Typhoon FLA 9500 gel was exposed to Fuji FLA-3000 Phosphor Screen. Efficiency of glycosylase activity was quantified using Image Gauge V4.0 software.

3 Results and Discussion

3.1 Study of human TDG futile activity towards oligonucleotide duplexes containing enhancers sequences

In our unpublished data, we have demonstrated that TDG exhibits futile excision activity towards regular thymine and cytosine opposite to undamaged adenine and guanine residues, respectively, in TpG/CpA and CpG/GpC, TpA/ApT contexts in duplex DNA. Here, we delve into substrate specificity of futile DNA glycosylase activity performed by TDG on DNA duplexes containing *Lhx2_c-MYC*, enhancer sequence from [19]. 30 mer long DNA duplex was constructed for this purpose (Fig. 1, Table 1). Note that this method only allows us to see the cleavage of the [^{32}P]-labeled DNA strand; it is not possible to identify the activity of DNA repair enzymes on the complementary non-labeled DNA strand. Because of this reason each DNA strands were labeled at 5' with γ[^{32}ATP] and hybridized with the corresponding non-labeled complementary strands, resulting in two labelled duplexes on which the action of TDG on both strands can be monitored. Then duplex oligonucleotides incubated at 37°C for 1h and 18h with human TDG^{FL} and followed by alkaline treatment to reveal the presence of abasic sites (Fig. 1).

According to what was anticipated from the earlier research we obtained excision of C and T from regular DNA, as you can see on lane 5 and 6 incubation with momo-functional glycosylase leads to the formation to cleavage fragments of 16 mer, 14 mer and 9 mer, corresponding to the cleavage of T at position 17 and 10 in TpG context, C at 15 in CpA context for *Lhx2_c-MYC* strand. Importantly no cleavage observed for T and C provided in the other contexts (CpT, TpT, TpC, etc.) and when DNA is incubated in the absence of enzyme.

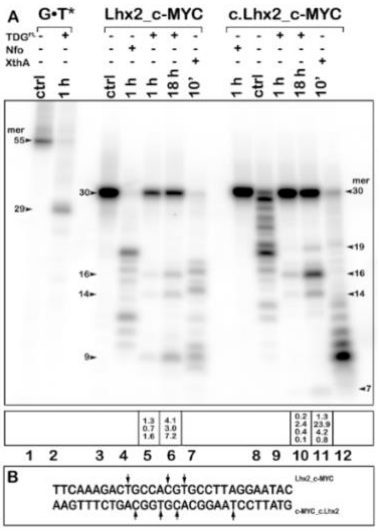

Fig. 1. Action of full-length hTDG on 30 bp oligonucleotide duplex *Lhx2_c-MYC* (A) Autoradiograph; (B) Sequence scheme *Lhx2_c-MYC* with complementary strands. Note: arrow indicates positions of T and C excised by TDG

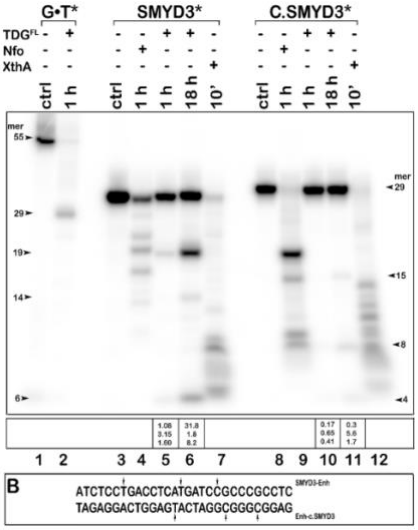

Fig. 2. Action of full-length hTDG on 29 bp oligonucleotide duplex *SMYD3* (A) Autoradiograph; (B) Sequence scheme of *SMYD3* with complementary strands. Note: arrow indicates positions of T and C excised by TDG

For the complementary *c.Lhx2_c-MYC* strand, we observe the same cleavage of T at position 17 and 8 in TpG and TpA contexts and at position 20 and 15 for C in a CpA and CpG contexts, which are validated by formation of 16 mer and 7 mer after T excision and 19 mer and 14 mer after cleavage of C. According to the cleavage efficiency results, we can assume that T and C are excised more efficiently in TpG (4,2%) and CpG (23,9 %) contexts compared to TpA (1,3%) and CpA (0,8%) contexts. Despite the presence of other TpG or CpA dinucleotide sequences on both strands, we do not observe their excision by TDG. Given the possible influence of CpGpTpG, as shown in Fig.1 B, this suggests that TDG cleavage is dependent on a sequence context covering more than two nucleotides in the middle of both strands. This highlights the preference for cleavage: either CpG or TpG individually are more preferred contexts for the enzyme, and neighboring C and T excisions are ignored because of this. Another possible explanation implies that there is not enough space for interaction of TDG with DNA if dinucleotides are positioned close to the ends (1-3 nucleotide) of strands as it is shown for *c.Lhx2_c-MYC* in Fig 1 B.

G•T* (“*” denotes labelled DNA strand with the residue of interest) at lanes 1-2 indicates positive control of TDG glycosylase classical activity and additional marker for underlining product sizes on the autoradiography results. Additional markers (lane 4,7,9 and 12) were obtained by dint of incubation of 5’-[^{32}P]-labeled oligonucleotides with Nfo1 and XthA nucleases for 1h and 10 min respectively.

Next, we examined another sequence called SMYD3, enhancer region of gene responsible for expression of chromatin modifier called (SMYD3) which has been connected to the emergence and spread of several cancer forms [16]. Here we used the same approach as in the previous results, by labelling both strands of this oligonucleotide duplex and incubating them in the presence of the enzymes mentioned below, followed by treatment with NaOH to cleave the AP sites. As expected, after 18 h incubation, TDG removes C and T from regular DNA in CpG and TpG contexts (lanes 5 and 6) on 5'-[^{32}P]-labelled *SMYD3* strand with 31.8% efficiency for C at position 20, and 1.8% efficiency for T at position 15 and 8.2%

for T at position 7 (Fig.2 A). For the complementary *c.SMYD3* strand, futile excision of bases was weaker with an excision rate of 1.7 % for T at position 16 and 5.6 % for C at position 9, and 1.7 % for C at position 5. The low efficiency of TDG-catalyzed excision on c.SMYD3 correlates with our unpublished data showing that in the presence of excision of base with high effiency, excision of T with greater than 30% efficiency (lane 6, 19 mer product) by TDG, neighboring futile activity inhibited in a sequence-specific independent manner.

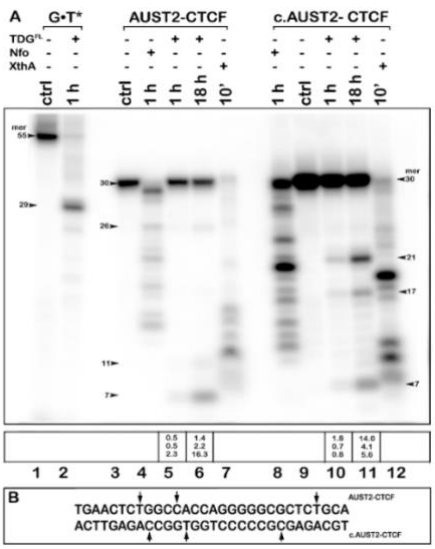

Fig. 3. Action of full-length hTDG on 30 bp oligonucleotide duplex *AUST2-CTCF.*
(A) Autoradiograph; (B) Sequence scheme *AUST2-CTCF* with complementary strands.
Note: arrow indicates positions of T and C excised by TDG

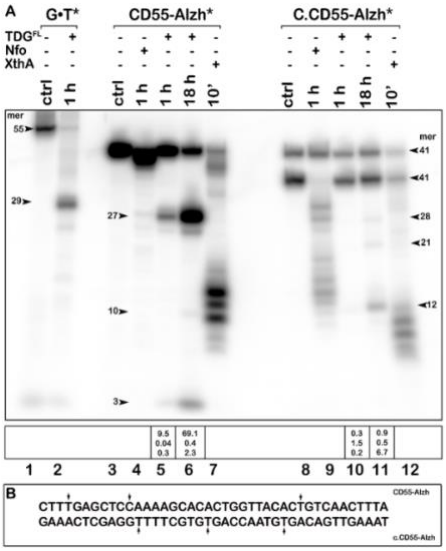

Fig. 4. Action of full-length hTDG on 41 bp oligonucleotide duplex *CD55-Alzh.*
(A) Autoradiograph; (B) Sequence scheme *CD55-Alzh* with complementary strands.
Note: arrow indicates positions of T and C excised by TDG

We further characterize the glycosylase activity of TDG towards AUST2-CTCF, Autism susceptibility candidate 2 (AUTS2) has become known to be a critical gene linked to a variety of neuropsychological conditions, including epilepsy, schizophrenia, ASD, and ID [20]. For this, the the reaction's byproducts were examined using denaturing PAGE. after hot alkaline treatment. As shown in Fig. 3, long incubation of 5'-[32P]-labeled AUST2-CTCF and c.AUST2-CTCF duplexes with TDG^{FL} produced multiple cleavage fragments (lanes 5, 6, 10, and 11). Predictably, TDG^{FL} generates cleavage products corresponding to the size of 26, 11, and 7 mers (lanes 5 and 6) suggesting excision of regular cytosine and thymine residues opposite to G in A in CpG, TpG, and CpA contexts. TDG^{FL} excises with 15 % efficiency C at position 21 opposite to G in CpA context in c. AUST2-CTCF strand and more than 16 % efficiency T at position 7 opposite to A in opposite TpG dinucleotide in AUST2-CTCF strand, as shown in Fig. 3 on lanes 6 and 11, suggesting that the futile DNA glycosylase activity in this sequence context pair established almost same excision rate.

Experiments with CD55-Alzh showed thymine removal at position 28 with relatively good (69, 1 %) efficiency after incubation for 18 h (Fig 4, lane 6). Non-cutting of nearby T or C may be inhibited by high affinity to the context mentioned above, which may be explained by the fact that after hydrolysis of the glycosidic bond between base and deoxyribose, TDG binds tightly to the AP site and prevents other TDG units from interacting with DNA at nucleotides neighboring the AP site bonded TDG. It could be due to the interaction of the disordered N- and C-terminal domains of TDG with DNA.

To check the possible involvement of TDG in formation C to T and T to C transitions in ADNP gene [17], we constructed oligonucleotide duplexes containing T at position 17 for substrate named *C>T-ADNP/c.C>T-ADNP* and C at the same position for substrate named *ADNP-T>C/c.ADNP-T>C*. Even after incubation for 18 h in the presence of TDG, no significant excision of T and C at the indicated positions was observed, as can be seen in Fig. 5, where the 16-mer fragments at lanes 6 and 21 were excised by only 1.4 % and 0.25 %, respectively. Overall, on the oligonucleotide substrates used, the efficiency of T and C excision catalyzed by enzyme activity was below 2% for all in the indicated contexts.

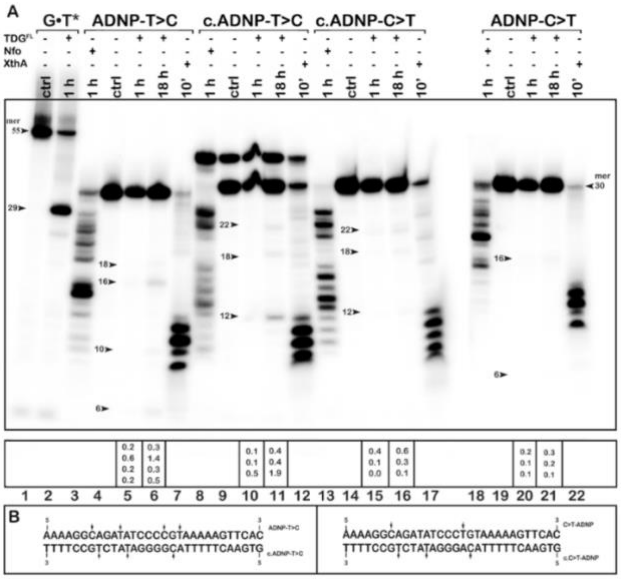

Fig. 5. Action of full-length hTDG on 30 bp *C>T-ADNP* and *ADNP-T>C* DNA duplexes. (A)Autoradiograph; (B) Sequence schemes *C>T-ADNP* and *ADNP-T>*C with complementary strands. Note: arrow indicates positions of T and C excised by TDG

4 Conclusion

It is tempting to hypothesize, given our findings, that the TDG enzyme appears to play a role in catalyzing the futile excision of regular, correctly matched pyrimidines from TpG/CpA, CpG and TpA contexts. This may contribute to the formation of single strand breaks (SSBs) within enhancer regions of neuronal cells. These SSBs can have a significant impact on gene regulation and neuronal function, potentially affecting the overall health and function of the nervous system. TDG-mediated futile repair in enhancer regions in neuronal cells can disrupt the regulatory mechanisms that control gene expression, potentially leading to abnormal neuronal development and function. In addition, the involvement of TDG in the formation of SSBs highlights the potential of this DNA glycosylase's function in neurodegenerative diseases and neurological disorders. An understanding of the precise mechanisms by which TDG is involved in this process may provide valuable insights into the pathogenesis of brain disorders and may offer potential therapeutic targets for intervention. Overall, further research is warranted to elucidate the specific consequences of TDG enzyme-catalyzed futile repair in neuronal cells and its potential contribution to neurodegenerative diseases.

Acknowledgments

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