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► **To cite this version:**

Jérôme Loc'h, Marc Delarue. Terminal deoxynucleotidyltransferase: the story of an untemplated DNA polymerase also capable of DNA bridging and templated synthesis across strands. *Current Opinion in Structural Biology*, 2018, 53, pp.22-31. 10.1016/j.sbi.2018.03.019 . pasteur-03414957

HAL Id: pasteur-03414957

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Submitted on 4 Nov 2021

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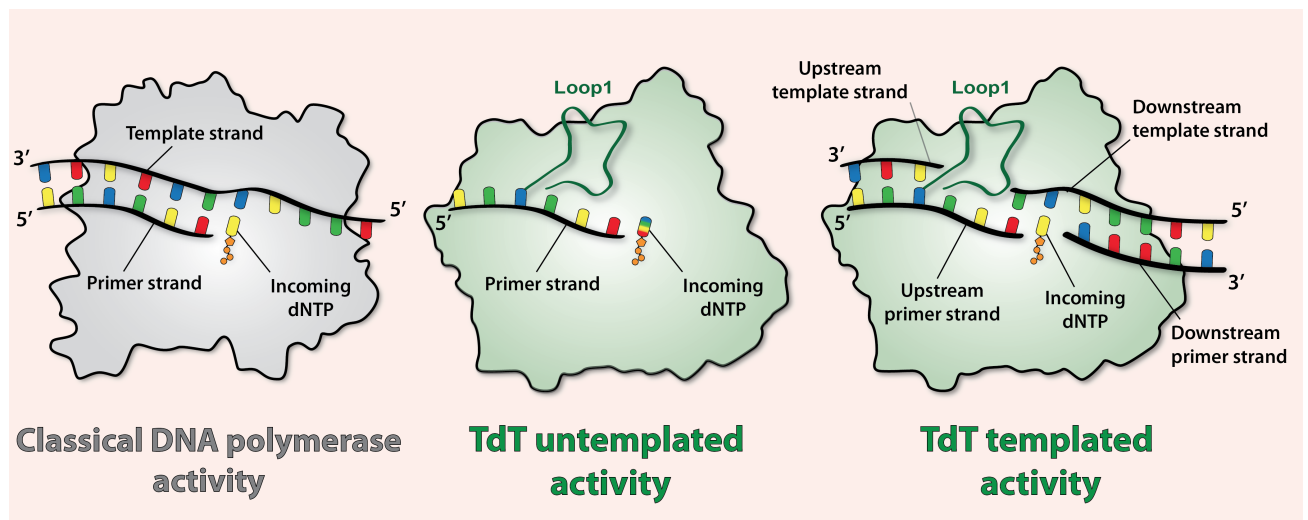
Terminal deoxynucleotidyltransferase: the story of an untemplated DNA polymerase capable of DNA bridging and templated synthesis across strands

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Summary

Terminal deoxynucleotidyltransferase (TdT) is a member of the polX family which is involved in DNA repair. It has been known for years as an untemplated DNA polymerase used during V(D)J recombination to generate diversity at the CDR3 region of immunoglobulins and T-cell receptors. Recently, however, TdT was crystallized in the presence of a complete DNA synapsis made of two double-stranded DNA (dsDNA), each with a 3' protruding end, and overlapping with only one micro-homology base-pair, thus giving structural insight for the first time into DNA synthesis across strands. It was subsequently shown that TdT indeed has an *in trans* template-dependent activity in the presence of an excess of the downstream DNA duplex. A possible biological role of this dual activity is discussed.

Bullet-points

- TdT has been known for more than 50 years as an untemplated DNA polymerase
- However, it was recently crystallized in a DNA bridging context and shown to bind a DNA synapsis in solution
- This is the first structure of a polX caught in the act of DNA synthesis across strands
- This dual activity of TdT can be understood in the context of its biological role.

Introduction

Terminal deoxynucleotidyltransferase (TdT) was one of the the first eukaryotic DNA polymerases purified in the early 1960s [1], from calf thymus extracts. However, instead of the expected classical *templated* polymerase activity, the biochemical characterization of TdT revealed an efficient *untemplated* polymerase (nucleotidyltransferase) activity [2,3], especially in the presence of divalent transition metal ions [4]. *In vivo*, the function of TdT was only fully understood in the eighties [5–7], after the discovery of V(D)J recombination [8–10]. During this process, TdT adds random nucleotides (N-segments) at the V-D and D-J junctions in heavy chains of immunoglobulins (Ig) and T-cell receptors, thereby contributing significantly to the diversity of the immune repertoire [11,12]. Subsequently, it was revealed that the V(D)J uses the same machinery [13,14] as the one of Non-Homologous End Joining (NHEJ) that repairs DNA double-strand breaks (DSB). This machinery includes a recognition complex (Ku heterodimer, DNA-PKcs), DNA end-processing enzymes such as a nuclease (Artemis or Metnase) and a DNA polymerase (pol X), as well as a ligation complex (Lig IV, XRCC4, XLF) [15]. The DNA polymerase is a member of the family polX that includes not only TdT, but also pol λ [16] and pol μ [17,18], the last two participating to both NHEJ and V(D)J recombination [19–23]. X-ray structures of all three polymerase domains have been determined to high resolution [24–26] but the only one that was crystallized in a DNA-bridging context is TdT [27,28]. Here we focus on TdT and on the biological implications of these new structures.

Structural and biochemical features of pol X family polymerases

The X-family DNA polymerase (polX) is specialized in DNA repair. This family is composed of four different DNA polymerases: pol β , pol λ , pol μ and TdT. Only three members of the polX family possess an N-terminal BRCT (BRCA1 carboxy-terminal) domain (**Figure 1A**) that is essential for NHEJ activity both *in vitro* and *in vivo* [29,30]. Pol β participates only in base excision repair (BER) [31,32] and is devoid of this small domain (around 11 kDa), which mediates protein-protein or protein-DNA interactions [33]. Three individual structures of a BRCT domain from the polX family were solved by NMR [34,35]. BRCT domain is typically an $\alpha\beta\alpha$ sandwich made up of a central antiparallel β -sheet flanked by three α -helices [36–38]. Although the structures of all known BRCT domains are highly conserved, their interaction mode with ligands is greatly variable and their role in the sequential recruitment of the different proteins during NHEJ repair is not completely understood [39]. Ligase IV contains 2 BRCT domains whose peptide junction interacts with XLF, XRCC4 [40,41] and which interact with Ku heterodimer [42]. The BRCT domain of pol μ binds DNA [43] but the precise mode of binding of the BRCT domain of either pol μ or TdT to Ku heterodimer remains to be elucidated, as well as its orientation with respect to the catalytic polymerase domain.

Sequence analysis of the polymerase domain of polX polymerases allows to divide this family into two sub-groups [44]. Pol β is closer to pol λ (34% of pairwise sequence identity) and TdT is closer to pol μ (44% of pairwise sequence identity). The structures of all four polymerases belonging to X-family have been solved by X-ray crystallography. Pol β was the first polX to be solved, alone and with different binary and ternary complexes [45–47]. The overall structure of the catalytic domain shares the same general architecture (but not the topology) of all DNA polymerases, namely a finger domain, a palm domain and a thumb domain (**Figure 1**). The

additional 8-kDa domain in pol β and pol λ contains a deoxyribose phosphate (dRP) lyase activity required in base excision repair (BER) of oxidative DNA damage (**Figure 1**). The amino acids necessary for dRP lyase activity are not conserved in TdT and pol μ , which do not participate in BER.

One striking feature common to all polX is the high degree of conservation of the catalytic site, with the three strictly conserved Aspartates (**Figure 1B**) coordinating two essential metal ions: in the two-metal ions mechanism, first described in [48] and later shown to be present also in DNA polymerases [47]: Metal A activates the 3'OH of the last nucleotide to allow the attack of the alpha phosphate while Metal B that comes in with the incoming nucleotide triphosphate stabilizes the departing group (PPi). In TdT, the coordination geometry of the divalent metal ions was studied in detail during a full catalytic site, including transition metal ions such as Mn^{++} , Co^{++} and Zn^{++} which are known to be more efficient than Mg^{++} for the nucleotidyltransferase activity, and it was concluded that Metal A has to leave and be replaced by Na^+ in order to allow translocation of the newly extended primer strand into a catalytically competent position for a new addition [49]. A movie of the reaction cycle, based on thirteen different structures was built [49]. This scenario was also described in even greater details for pol β where it was also found that binding of Na^+ in Metal A binding site, after nucleotide incorporation, is a key step for DNA translocation [50]. In addition, time-lapse crystallography showed that in pol β , there is an additional divalent ion (a third Mg^{++}) that comes during the reaction to counter-balance the apparition of a charge on the beta phosphate, and then leaves before the end-state is reached [50,51]. This situation was also observed for pol μ , in the case of Mn^{++} ions [52].

Common features of TdT, pol μ and pol λ

TdT, pol μ and pol λ remain in a closed conformation throughout their catalytic cycle, contrary to pol β [24,53,54]. One possible explanation for this observation is that they have traded fidelity (which requires open-to-closed transition) for a very tight binding of the DNA synapsis, a very fragile structure. One unique feature revealed when the first x-ray structure of TdT was solved [24], is a specific Loop (Loop1), composed of 20 amino acids (382-401), located between the β 3 and β 4 strands (**Figure 1B**), that prevents the binding of a 5' overhang of the template strand. More than 30 structures of TdT (wild-type or mutants in different complexes) are available on PDB and in all of them, Loop1 adopts the same lariat-like conformation that prevents the binding of an *uninterrupted* template strand on TdT (**Figure 2**). Perhaps somewhat deceptively, in all known structures of pol μ , Loop1 (also about 20 amino acids long) is invisible in the electron density map, meaning that this region is disordered (**Figure 2 and 3A**). In pol λ , Loop1 is comparatively shorter (8 aa), but still longer than in pol β (**Figure 3**). However, pol λ has an additional Loop, called Loop3, that, interestingly, is located precisely where another form of TdT resulting from alternative splicing has an insertion of 20 additional residues [55,56] (**Figure 3B**) and where it is ideally placed to control bulges or insertions just before the *in-trans* templating base [28] (**Figure 3A**). Mutations experiments have consistently shown the importance of Loop1 for the substrate specificity not only in TdT [27,57] but also in pol μ [58–60], as well as pol λ [61].

Early sequence comparisons in a structural context helped to define two important regions for the specificity of TdT vs pol μ [24], later named SD1 and SD2 [57] (**Figure 3**): they are located at the C-terminal border of Loop1, and in a β -turn- β structure close to Loop1, which can also bind an extra Zn^{++} ion [49] but the precise

role of this additional divalent ion is currently unknown. Mutations in these two regions profoundly affect the activity of both TdT [27,28] and pol μ [60]. Mutation of only one amino acid in SD1 region (F401A) confers to Tdt an *in cis* templated polymerase activity, even in the presence of Co^{++} [57].

A remaining puzzle concerns the conformation of Loop1 in pol μ , and its role in binding the DNA synapsis substrate. Experiments are currently underway in our lab using a TdT-pol μ chimera to determine the conformation of Loop1 of pol μ and they indeed suggest that Loop1 plays the crucial role of a gate that can be open or closed (disordered or ordered) when pol μ is bound to its biologically relevant substrate, namely a DNA synapsis (Loc'h et al., submitted).

Nucleotidyltransferase activity of TdT

Extensive biochemical experiments have demonstrated that TdT can add random deoxyribonucleotides (dNTPs) on a ssDNA primer, which has to be at least three nucleotides long, in a template-independent manner [2]. *In vitro* experiments show that TdT can use all four natural dNTPs with a preferential incorporation of dCTP and dGTP compared to dATP and dTTP [3]. Pol μ also has a significant nucleotidyltransferase activity in the presence of Mn^{++} [57,62,63]. Interestingly, TdT nucleotide binding site can accommodate both deoxyribo-nucleotide and ribo-nucleotide triphosphates (dNTPs and rNTPs). TdT shares this property with pol μ , but not with pol λ and pol β (**Figure 1A**). Indeed, the presence of a YF sequence motif, the so-called steric-gate at the vicinity of the 2' OH of the nucleotide, prevents the binding of rNTPs in pol λ and pol β , whereas a GW sequence motif in TdT and pol μ (**Figure 1B**) increases the size of the nucleotide binding pocket, allowing binding of both dNTPs and rNTPs [64,65]. However, addition of rNTPs by TdT stops after few

incorporations on ssDNA [66,67]. This can be interpreted by noting that the path of the primer is constrained in a B-DNA form by the protein, especially through a Na⁺ ion coordinated by the HhH2 motif (**Figure 1B**) at the level of the penultimate phosphate of the primer, and this B-DNA form is not suitable for an RNA backbone. TdT can also incorporate efficiently various un-natural bases [68,69]. An interesting consequence of the large tolerance of TdT on the incoming nucleotide is to use it for making polymers of un-natural DNA, using nucleotides modified either in the sugar moiety or the base moiety [70] or for click chemistry [71]. Also a recent application for FISH experiments and the design of RNA capture probes can be found in [72].

***In trans*-templated polymerase activity across strands breaks**

In 2015-2016, half a century after its first biochemical characterization as a template-independent polymerase, it was shown that TdT can i) assemble a DNA synapsis by itself, optimally with one micro-homology base-pair between strands [27] and ii) perform a template-dependent nucleotide incorporation across strands breaks [28] in the presence of an excess of downstream dsDNA with a 3' protruding end. Because this template-dependent activity of TdT is achieved by using an *in trans* template strand, instead of the usual *in cis* template strand, we refer to it as the *in trans* activity. Interestingly, this *in trans* templated activity was also described for pol μ [60,73], but without the need of an excess of the downstream DNA duplex [28]. *In vitro* biochemical experiments on *chimeric* constructs of TdT, involving substitution of Loop1 by pol μ 's sequence and/or reconstitution of the 5'-phosphate binding site (**Figure 1**), show an activity similar to pol μ , with a protein/DNA ratio of 1:1 [28]. The existence of templated synthesis across strand breaks has recently been described *in vivo* for both pol μ and pol λ [74–77].

The structure of TdT in complex with a DSB-DNA substrate [28] is the first of its kind to be solved for a polX (**Figure 2**). It looks as if TdT was designed to “isolate” a mini-helix made of only two base pairs to stabilize and establish a fragile bridge between the upstream and downstream duplexes (Figure 1). The two upstream and downstream dsDNA are in B-DNA conformation, while the micro-homology (MH) mini-helix between them is in A-DNA conformation. L398 in Loop1 is crucial to break the helical path from the upstream dsDNA to the MH-mini helix and its role has been verified by site-directed mutagenesis [27].

Function of polX during V(D)J recombination

Expression of TdT is only observed in the primary lymphoid organs, thymus and bone marrow where V(D)J recombination is active [78]. Indeed, expression of TdT is only detected during heavy chain rearrangements, but is absent from the next step where light chain rearrangements occurs [79] (**Figure 4**). The expression of TdT is also tightly regulated in time as it is not expressed in fetal or neo-natal life. One way this regulation is done is probably through ubiquitylation [80]. Interestingly, two TdT interacting factors (Tdif1 and Tdif2) have been identified and characterized to inhibit Tdt activity [81,82]. Pol μ participates in light chain rearrangements during V(D)J recombination, whereas pol λ participates only in heavy chain rearrangements [83] (**Figure 4**). It should be noted that when TdT is made to express in non-lymphoid cells, it participates in NHEJ DNA repair [84]. Also, when expressed constitutively in B-cells, it generates N-regions in both heavy and light chains [85].

It was found that inhibiting TdT in some cancer cells can kill them, such as in acute lymphoblastic leukemia cells [86]. Chemical compounds were designed and

synthesized using TdT as a drug target [87,88] and the structure of some of these compounds was solved in their bound form [89].

The length of the N-segments incorporated by TdT ranges from 2 to about 15-20, with two clearly different regimes in its probability distribution function: a rising phase with a peak at 4, followed by a decreasing phase [90,91]. Loc'h and colleagues proposed that the dual activity of TdT may correspond to these two regimes [28]: after the addition of a few random nucleotides on the 3' end DNA, the downstream DNA is finally reached, at which point TdT switches to an *in-trans* template-dependent synthesis (**Figure 4**). This synthesis will stop if the microhomology base pair is of Watson-Crick type and continue otherwise, which occurs in three out of four possible cases; strikingly, this quantitatively explains the size-distribution law of the N-regions of the second phase, that exponentially decreases with a slope of $-1/4$ [91].

After incorporation of random nucleotides by TdT during heavy-chain rearrangements, both TdT and pol λ may perform *in-trans* polymerase activity (in unknown proportions), whereas synthesis of the complementary strand can only be achieved by pol λ using its gap-filling activity (**Figure 4**). In light-chain gene rearrangements pol μ can perform not only template-independent, but also *in-trans* polymerase and gap-filling activities (**Figure 4**).

Perspectives and Conclusion

Due to its ability to add random sequences to a DNA primer, TdT is obviously an “unpredictable” polymerase (rather than a “misguided” one [12]), which explains its tight regulation both in time and in space. Here we suggest that its gradual stop is also programmed, by switching to a previously unsuspected templating mode across

strand breaks, due to the spatial constraints of the architecture of the whole NHEJ apparatus. Significant progress has been made recently in the structural biology of the ligation complex [92], as well as the huge loading complex of NHEJ [93–95]. It is known that TdT interacts with Ku heterodimer through its BRCT domain, as does pol μ [13,14]. If the interaction of the BRCT domain with Ku heterodimer could be mapped, then it would be possible to place the polX with respect to the DNA-PKcs-DNA complex. On the evolutionary level it would be interesting to know how similar is the positioning of TdT and pol μ in this integrated view of the NHEJ complex. From Figure 1, it appears that TdT has lost just the 5' phosphate binding site of pol μ and its Loop1 is of the same length, but with a different sequence. The structure of the TdT chimera containing Loop1 of pol μ will inform us on its conformation and allow a comparison with the LigD polymerase that performs NHEJ in bacteria.

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First structure of a ternary complex of a polX with a gapped-template DNA substrate and the incoming nucleotide, which sets up the stage for all polX polymerases and describes for the first time in atomic detail the coordination of the two essential catalytic ions through strictly conserved Aspartates. An open-to-closed transition is described. A landmark paper for polX.

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49. * Gouge J, Rosario S, Romain F, Beguin P, Delarue M: **Structures of intermediates along the catalytic cycle of terminal deoxynucleotidyltransferase: dynamical aspects of the two-metal ion mechanism.** *J Mol Biol* 2013, **425**:4334–4352.

Binary and ternary structures of TdT in precatalytic, post-catalytic and competent states. It is demonstrated that some of the transitions between these states can occur in the crystal. The necessity of metal A departure for DNA translocation and new nucleotide binding is deduced and a movie of the catalytic cycle is built from 13 different structures. A change of the coordination of Cobalt⁺⁺ from tetrahedral to octahedral during the reaction is described. The presence of an additional Zn⁺⁺ near Loop1 is also demonstrated.

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Comment on [50].

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58. * Juárez R, Ruiz JF, Nick McElhinny SA, Ramsden D, Blanco L: **A specific loop in human DNA polymerase mu allows switching between creative and DNA-instructed synthesis.** *Nucleic Acids Res* 2006, **34**:4572–4582.

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60. * Martin MJ, Blanco L: **Decision-making during NHEJ: a network of interactions in human Pol μ implicated in substrate recognition and end-bridging.** *Nucleic Acids Res* 2014, **42**:7923–7934.

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Legends of Figures

Figure 1

A. Domain organization of eukaryotic polX family; the pol IV of *S. cerevisiae* and *S. pombe* have been omitted. B. Three-dimensional structure of TdT bound to a DNA synapsis [28]. The color code for essential specific features (HhH motif, 5'-phosphate binding site, Loop1, steric gate) is the same as in panel A. The catalytic aspartates are shown in ball-and-stick representation, as well as residues from the steric gate (GW) and the HhH2 motif (GVG). The DNA synapsis is in cyan, the 3' end of the primer strand is in pink, the catalytic divalent ions are shown as CPK spheres (in green), the Na⁺ ion bound to the HhH2 motif and a phosphate of the primer strand is in blue.

Figure 2

Schematic representation of all available structures for TdT, pol μ , pol λ and pol β : the apo form, complexes with the incoming dNTP, with a downstream (D/S) dsDNA substrate, a gap-filling DNA substrate or a true DNA synapsis. Loop1 conformation is highlighted, when visible in the electron density map. The DNA substrates are explained in the top panel.

Figure 3

A. Superposition of the DNA synapsis region in the four known polX-DNA complexes, highlighting the conformations of Loop1 and Loop3, as well as the position of the motifs SD1 (purple) and SD2 (dark blue). TdT is in green, pol μ is in blue, pol λ is in brown and pol β is in red.

B. Multialignment of murine sequences of TdT (with its two main forms resulting from alternative splicing), pol μ , pol λ and pol β , in the region of Loop1 and Loop3. The essential Sequence Determinants motifs [24], that maximally discriminate TdT and pol μ [27,57] are highlighted and referred to as SD1 and SD2.

Figure 4

Proposed scenario of the different DNA polymerase activities occurring at a V(D)J junction site for light-chain rearrangements (right panels) and heavy-chain rearrangements (left panels). From top to bottom: template-independent activity, *in trans* template-dependent activity and gap-filling activity. A putative arrangement of the DNA-PK complex is shown in the background, merely to indicate the scales. The ligation step by Ligase IV is omitted for clarity. The definition of the V, D, J regions, constant and variable regions in light chains and heavy chains is recalled in the central panel.

