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# The role of catalytic and regulatory domains of human PrimPol in DNA binding and synthesis

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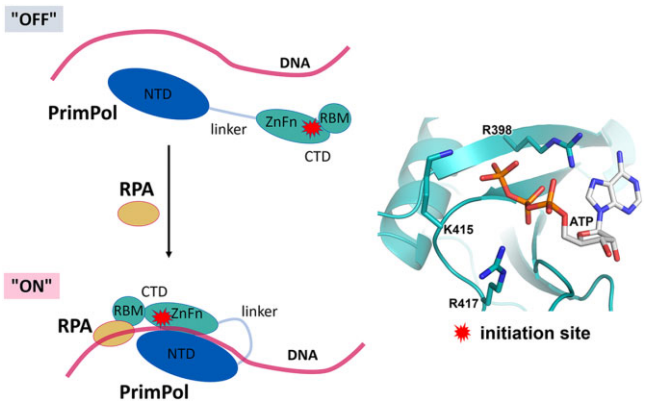
## ABSTRACT

**Human PrimPol possesses DNA primase and DNA polymerase activities and restarts stalled replication forks protecting cells against DNA damage in nuclei and mitochondria. The zinc-binding motif (ZnFn) of the C-terminal domain (CTD) of PrimPol is required for DNA primase activity but the mechanism is not clear. In this work, we biochemically demonstrate that PrimPol initiates *de novo* DNA synthesis in *cis*-orientation, when the N-terminal catalytic domain (NTD) and the CTD of the same molecule cooperate for substrates binding and catalysis. The modeling studies revealed that PrimPol uses a similar mode of initiating NTP coordination as the human primase. The ZnFn motif residue Arg417 is required for binding the 5′-triphosphate group that stabilizes the PrimPol complex with a DNA template-primer. We found that the NTD alone is able to initiate DNA synthesis, and the CTD stimulates the primase activity of NTD. The regulatory role of the RPA-binding motif in the modulation of PrimPol binding to DNA is also demonstrated.**

## GRAPHICAL ABSTRACT




## INTRODUCTION

Human primase polymerase PrimPol, belonging to the archaeo-eukaryotic superfamily, was described in 2013 (1–3). PrimPol is encoded by the *PRIMPOL* gene located on chromosome 4 at locus 4q35.1, and is a protein consisting of 560 amino acid residues with a molecular mass of 65 kDa (1,4). In contrast to the human replicative primase heterodimer PriS/PriL that synthesizes 9-mer RNA primers (5), PrimPol engages in *de novo* synthesis using deoxyribonucleotides, resulting in DNA primers that do not require removal.

PrimPol is present in both the nucleus and mitochondria (1). PrimPol primase activity allows for replication reinitiation at DNA sites containing damage or at secondary structures that block high-fidelity DNA polymerases (6–9). Lack of PrimPol in the cell leads to a slowing down of replication, chromosomal aberrations, and increased sensitivity to various DNA-damaging agents (1–3,10,11).

PrimPol is composed of two domains: an N-terminal AEP-like catalytic domain (NTD), and a C-terminal

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domain (CTD) similar to the UL52 primase of the herpes simplex virus (Figure 1) (4,12). Two crystal structures of the human NTD (residues 1–354) in complex with a primer/undamaged DNA template and with a primer/DNA template with 8-oxo-G and incoming nucleotides have been deciphered (PDB ID: 5L2X; PDB ID: 7JK1) (13,14). The active site of the NTD is formed by the N-helix (residues 1–17) and two modules: ModN (residues 35–105) and ModC (residues 108–200 and 261–348). Conservative motifs I (DxE) and III (hDh) of ModC contain the key catalytic amino acid residues Asp114/Glu116 and Asp280 involved in the coordination of $Me^{2+}$ ions, while motif II (SxH) contains Ser167 and His169 residues that bind the incoming nucleotide (13). Mutations of Asp114/Glu116, Asp280 and His169 residues result in loss of DNA polymerase and primase activities of human PrimPol (1–3,15).

Conserved motifs Ia (RQ) (residues Arg47 and Gln48) and Ib (QRhY/F) (residues Gln75, Arg76 and Tyr78) of the ModN module form contacts with a DNA template (13). Mutations of the Arg47 and Arg76 residues significantly reduce the DNA polymerase and primase activities of PrimPol (16). The N-helix is connected to ModN by a long flexible linker (residues 18–34) and interacts with the DNA template strand by making a few contacts with the major groove (13). Unlike DNA polymerases, the NTD of PrimPol is almost completely devoid of contacts with a primer, and its active site has room to accommodate the initiating NTP to form a dinucleotide during *de novo* DNA synthesis (13).

The CTD domain contains a conserved C–H–C–C zinc finger (ZnF) motif that coordinates the $Zn^{2+}$ ion (~372–487 residues harboring Cys419, His426, Cys446, Cys451) (7,12,17). It was shown that ZnFn is required for *de novo* DNA synthesis but not for DNA polymerase activity. Deletion of ZnFn and mutations of the conservative residues Cys419 and His426 that coordinate zinc disrupt PrimPol primase activity while retaining DNA polymerase activity (3,7,12,17).

Amino acid residues 201–260 of the ModC module represent an unstructured region and presumably play a regulatory role. Residues 226–232 form contacts with the accessory protein PolDIP2 (18,19). Residues 60–70 of the ModN domain of PrimPol form the second binding site for the replicative factor PolDIP2 (19). The CTD contains the RPA-binding domain (RBD) harboring two negatively charged RPA-binding motifs (RBM): RBM-A (residues 513–527), and RBM-B (residues 546–560) (20).

The detailed mechanism of PrimPol primase activity is not fully understood. Structures of the full-length PrimPol and the initiation complex of PrimPol with a DNA template and a dinucleotide have not been deciphered. However, some important information about the mechanism of PrimPol primase activity has been obtained from biochemical studies. PrimPol prefers to initiate DNA synthesis on the 3′-GTC-5′ sequence with cryptic G (1,21), and its activity is dependent on cofactor metal ions. PrimPol exhibits DNA polymerase activity in the presence of $Mn^{2+}$ and $Mg^{2+}$ ions, but primase activity is stimulated exclusively by $Mn^{2+}$ ions (15,22). It has been suggested that after binding and recognizing the preferred DNA site, PrimPol binds the first nucleotide at the elongation site, which is stabilized by $Mn^{2+}$ ions (17). It is assumed that binding of the second nucleotide (preferably ATP) occurs at the initiation site, followed by catalysis and formation of the 5′-rA-dC dinucleotide.

According to the suggested model, there is a division of work between the catalytic subunit/domain and the accessory subunit/domain (often containing ZnF or an iron-sulfur cluster) in primases (5). In human primase, the small catalytic PriS subunit is responsible for catalysis, whereas the flexibly tethered large accessory PriL subunit is responsible for the binding of a template and initiating NTP. The interaction of PriL with the RNA/DNA hybrid involves the CTD and the 5′-triphosphate of an RNA primer (5). In a similar way, dinucleotide formation with adenosine triphosphate is 16 times more efficient than with adenosine di- and monophosphates, suggesting that a triphosphate group at the 5′-initiator nucleotide is required for *de novo* DNA synthesis by PrimPol (17). Moreover, the elongation of a dinucleotide by PrimPol is possible only in the presence of a 5′-triphosphate on the initiating nucleotide (17). Deletion of the ZnFn suppresses dinucleotide formation with ATP and impedes further primer elongation, which suggests the ZnFn motif plays a role in the stabilization of ATP at the 5′-initiator site and/or in the coordination of cryptic Gua of a DNA template (17,23).

Due to bi-modal organization, primases can operate in the *cis*- or *trans*-orientation (Figure 1B). The *cis*-mechanism implies that the catalytic subunit/domain and accessory subunit/domain of the same molecule are involved in catalysis. In the *trans*-orientation, the accessory unit of one primase molecule binds DNA and/or the initiator nucleotide, while dinucleotide formation takes place in the active site of another molecule. DNA primase of phage T7 (24) and primase RepB′ (25) are able to initiate primer synthesis in the *trans*-mode, while human primase is not (26,27). The mechanism of PrimPol operation is currently unknown.

In the present work, we analyzed the mechanism of PrimPol primase activity using its variants that selectively disrupt catalytic DNA-synthetic function or only priming activity, as well as the individual catalytic domains of PrimPol. We show that PrimPol performs primer synthesis in the *cis*-orientation, when the NTD catalytic and CTD regulatory domains of the same molecule take part in catalysis. We also demonstrate a key regulatory role of the CTD in template-primer binding by PrimPol.

## MATERIALS AND METHODS

### Protein purification

The wild-type PrimPol and all mutant variants fused at the N-terminus with a SUMO-$HIS_{10}$ tag were purified from *Escherichia coli* cells as described (28). Human RPA was purified according to (29).

### DNA substrates

Oligonucleotides were synthesized by Eurogene and Syntol (Moscow, Russia). To prepare the DNA substrate for testing DNA polymerase activity, Primer-18 was 5′-labeled with [γ-$^{32}$P]-ATP by T4 polynucleotide kinase (SibEnzyme, Russia)

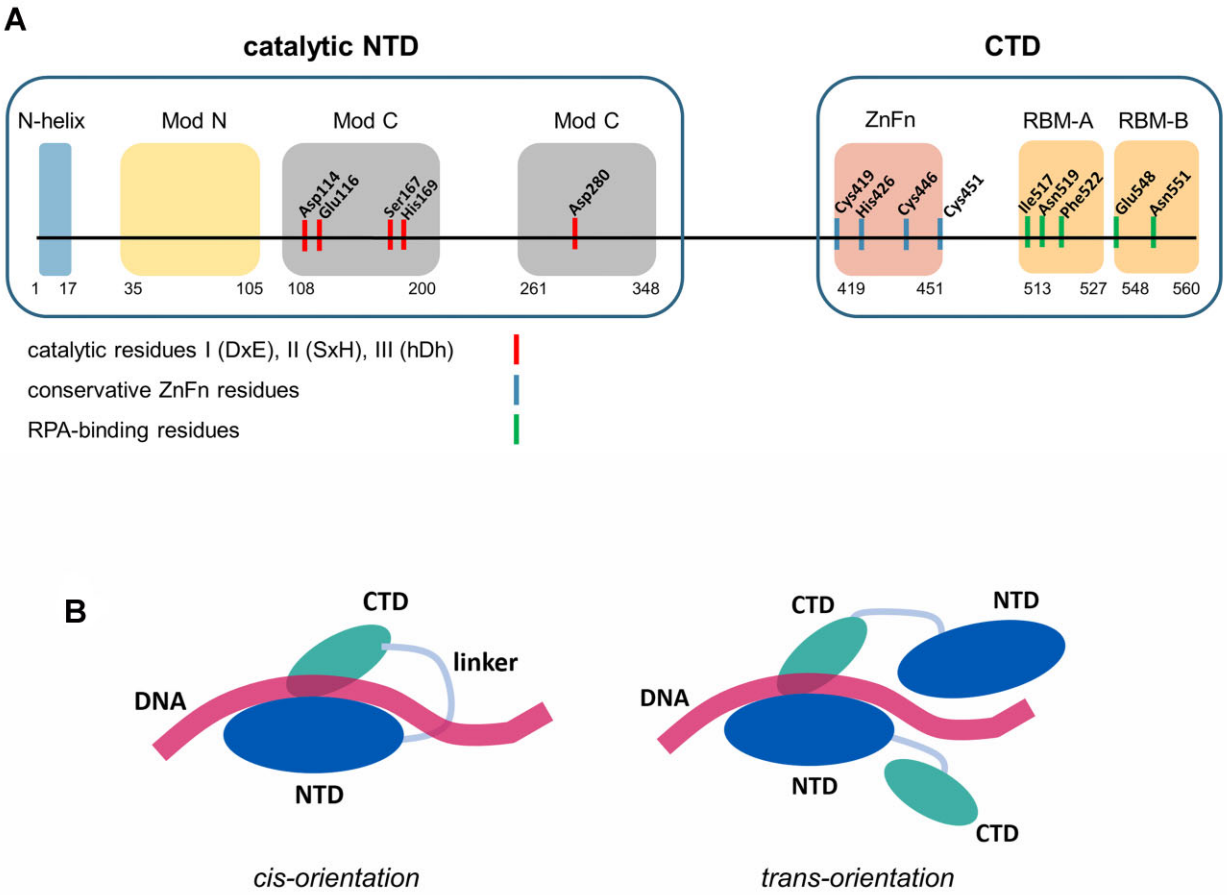


**Figure 1.** (**A**) Domain organization of PrimPol. The positions of the catalytic residues coordinating $Me^{2+}$ ions (Asp114, Glu116, Asp280), the conservative C-H-C-C ZnFn motif, and the RPA-binding motif residues are indicated. (**B**) The schematic representation of the *cis*- and *trans*-orientation of PrimPol domains during *de novo* synthesis.

and annealed to the corresponding unlabeled Template-55 at a molar ratio of 1:1.1, heated to 75°C, and slowly cooled down to 24°C. The sequences of the oligonucleotides used in this study are shown in Table 1. Oligonucleotide '*p-p-p*-12 containing the 5′-terminal adenosine triphosphate was synthesized enzymatically *de novo* using wild-type PrimPol. 600 μl of reaction mixture (aliquoted in 6 tubes at 100 μl) contained 40 mM HEPES pH 7.0, 8% glycerol, 50 μg/ml BSA, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 10 μM 'Acctg24' template oligonucleotide, 200 μM dGTP and dTTP, 1 mM rATP, and 5 μM PrimPol. The reactions were incubated for 4 h at 30°C, and 2.5 μM fresh PrimPol was added to the reaction every hour. DNA was precipitated from solution by ethanol. Four volumes of ice-cold 96% ethanol were added to 1 volume of solution containing 300 mM NaCl, 10 mM $MgCl_2$, and 1 μg/μl glycogen and incubated overnight at –20°C. After centrifugation for 30 min at 21 500 g and 0°C, the precipitate was washed with 70% ice-cold ethanol, dried, and dissolved in 35 μl of $H_2O$. Next, an equal volume of loading formamide mixture (95% formamide, 20 mM EDTA) was added and tubes were heated at 95°C for 5 min. The synthesized product and template were separated on denaturing 16 or 20% PAGE with 7 M urea in 1× TBE. DNA imaging was carried out by the UV shadowing of DNA spots on a fluorescent thin-layer chromatographic Silufol plate (Supplementary Figure S1). Bands were excised from the gel, crushed, and extracted in 1 ml of extraction buffer (500 mM $NH_4Ac$, 10 mM $MgAc_2$, 1 mM EDTA, pH 7.0) overnight with shaking. DNA was directly precipitated from solution by ethanol and dissolved in 35 μl of $H_2O$. Alternatively, DNA was extracted in 1 ml of 100 mM NaCl with 5 mM EDTA and isolated on the QAE-Sephadex A-25 (Pharmacia Fine Chemicals) resin. 2–10 mer oligonucleotides with the 5′-terminal adenosine triphosphate were synthesized using DNA templates of corresponding length as described for the '*p-p-p*-12'.

**Table 1.** Oligonucleotides used in the study

| Name | Sequence (5′→3′) |
|---|---|
| Acctg24 | AAAAAAAAA**ACCTG**AAAAAAAAA |
| Acctg55 | AAAAAAAAAAAAAAAAAAAAAAAAAAA<br>AAAAAAAAAAAA**ACCTG**AAAAAAAAA |
| Cy5-Acctg | Cy5–AAAAAAAAA**ACCTG**AAAAAAAAA |
| Primer-18 | CGGTATCCACCAGGTCTG |
| Template-55 | GACTACATTTCATCTGGCTTGGGCTTCATC<br>GTTGTCGCAGACCTGGTGGATACCG |

### Primase reactions

DNA primase activity was tested in 6 μl reaction mixtures containing 40 mM HEPES pH 7.0 (or 7.4), 8% glycerol, 50 μg/μl BSA, 1 mM $MnCl_2$, 2 μM unlabeled oligonucleotide DNA substrate, 200 μM each of dGTP, dCTP and dTTP or dGTP alone, 10 μM dATP, 30 nM [$\gamma$-$^{32}$P]-ATP and 2 μM PrimPol. Test tubes were preincubated on ice for 5 min and reactions were started by dNTP and incubated at 30°C for 60 min or for the indicated time intervals. The reactions were stopped by adding an equal volume of loading formamide mixture. Experiments were repeated 2–4 times for each protein preparation. The synthesized products were separated on denaturing 30% PAGE with 7 M urea in 1xTBE and visualized on a Typhoon 9400 (GE Healthcare, USA).

### DNA polymerase reactions

Primer extension reactions were performed in 20 μl reaction mixtures containing 20 nM radioactively labeled oligonucleotide substrate, 200 μM of each dNTP, 40 mM HEPES pH 7.0, 8% glycerol, 50 μg/ml BSA, 100 nM PrimPol and 10 mM $MgCl_2$. Some reactions were preincubated with 10 nM RPA at 30°C for 3 min as indicated in the figure legend. Test tubes were preincubated on ice for 5 min and reactions were started by dNTP and incubated at 37°C for the indicated times. The reactions were stopped by adding an equal volume of loading formamide mixture. Experiments were repeated twice for each protein preparation.

### EMSA

Binding of PrimPol to the $^{32}$P-labeled Template-55/Primer-18 substrate, the fluorescently labeled ssDNA substrate Cy5-Acctg24, and the DNA template-primer substrate with *p-p-p*-12 annealed to Cy5-Acctg24 was performed in a 20 μl reaction mixture containing 40 mM HEPES pH 7.0, 50 mM KCl, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 1 mM $MnCl_2$, 300 nM DNA substrate, and 250–1200 nM PrimPol. Some reactions were supplemented with 1 mM ATP and 200 μM of each dNTP as indicated in the figure legends. The reactions were incubated at 24°C for 20 min and placed on ice. To analyze the effect of RPA, 25 nM RPA was preincubated with 50 nM $^{32}$P-labeled Acctg55 substrate at 8°C for 30 min to ensure complex formation prior to addition of PrimPol. Next, 300–3000 nM PrimPol or 50–200 nM $PrimPol_{1\text{-}475}$ variant were added on ice and tubes were incubated another 30 min at 8°C. Reaction mixtures were directly applied to a 5% native PAAG, and complexes were separated from free DNA in 0.5x Tris-glycine buffer (12.5 mM Tris, 96 mM glycine, pH 8.3) at 10 V/cm and at 4°C. The gel was visualized on a Typhoon 9400 (GE Healthcare, USA).

## RESULTS

### PrimPol operates in *cis*-orientation

To analyze the mechanism of primase activity, we replaced the key amino acid residues of the NTD and CTD of the full-length PrimPol: D114A substitution of the Asp114 residue coordinating catalytic $Me^+$ ions ($PrimPol_{D114A}$), and R417A and R424A substitutions of conserved ZnFn motif residues that presumably bind the 5′-triphosphate of initiating ATP ($PrimPol_{R417A}$ and $PrimPolR_{424A}$) (Figure 2A). In addition to these separation-of-function mutations, we also analyzed variants of PrimPol with deletions of the NTD ($PrimPol_{363–560}$) or CTD ($PrimPol_{1–363}$ and $PrimPol_{1–363CD}$) and RBM ($PrimPol_{1–475}$) (Figure 2A).

The DNA polymerase activity (Figure 2B), total DNA primase activity and dinucleotide formation (Figure 2C) of PrimPol variants and their combinations were analyzed. The D114A substitution resulted in the loss of any catalytic activity (Figure 2B, lanes 13–15 and Figure 2C, lanes 5, 11). The full-length PrimPol variants with ZnFn substitutions R417A and R424A retained DNA polymerase activity while their primase activity was significantly affected, especially for the R417A mutant (Figure 2C, lanes 3, 4, 9, 10). These data indicate that R417 and R424 are important only at the initial steps of DNA synthesis. This is consistent with previous studies showing the key role of the CTD in DNA synthesis initiation but not elongation (3,7,12,17,23).

In the case of the *trans*-mechanism of PrimPol, mixing in one reaction the two mutant forms $PrimPol_{D114A}$ and $PrimPol_{R417A}$ or $PrimPol_{D114A}$ and $PrimPol_{R424A}$ would result in the formation of functional dimer molecules carrying one functional catalytic NTD domain and one functional regulatory CTD. As a result of the cooperation between the functional domains of different mutant forms, the partial restoration of DNA-primase activity was expected. However, when mutant variants $PrimPol_{D114A}$ and $PrimPol_{R417A}$ or $PrimPol_{D114A}$ and $PrimPol_{R424A}$ were mixed in the reaction, no restoration of the DNA primase activity occurred (Figure 2). On the contrary, a slight decrease of all activities was observed in these reactions compared to reactions with $PrimPol_{R417A}$ and $PrimPol_{R424A}$ variants. The inhibition may result from the competition of catalytically active ZnFn-mutant variants with the catalytically inactive $PrimPol_{D114A}$ variant for a DNA substrate. These results are consistent with the model of the *cis*-orientation when the catalytic NTD and regulatory CTD of the same PrimPol molecule are involved in DNA synthesis.

### The NTD retains weak primase activity and cooperates with the separated CTD

Remarkably, the full-length $PrimPol_{R417A}$ and $PrimPol_{R424A}$ variants demonstrated levels of DNA polymerase activity similar to the wild-type enzyme (Figure 2B, lanes 16–18, 22–24), but deletion of the CTD (variant $PrimPol_{1–363}$) dramatically increased the DNA polymerase activity (Figure 2B, lanes 4–6). Moreover, separate NTD retained weak DNA primase activity (Figure 3A).

Interestingly, the DNA primase activity of the $PrimPol_{1–363}$ variant was higher at pH 7.5 compared to 7.0 (Supplementary Figure S2). This is contrary to the full-length PrimPol showing higher DNA polymerase (30) and DNA primase (Supplementary Figure S2) activity at pH 7.0 versus pH 7.5. The difference may be due to different isoelectric points of PrimPol (5.14) and NTD (7.61) or to a different mode of initiating NTP coordination in the absence of CTD. The absence of impurities and an extrinsic

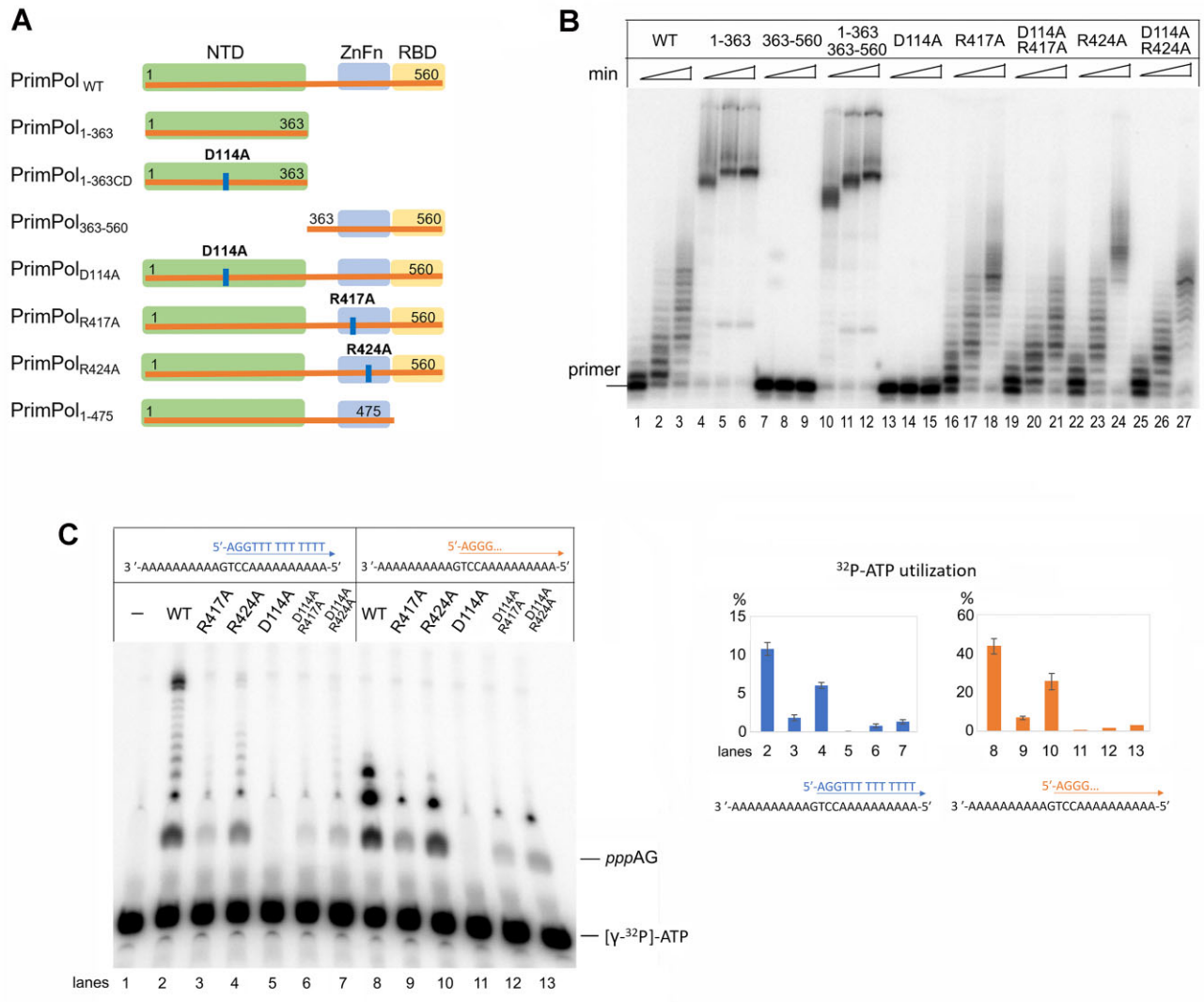


**Figure 2.** The DNA polymerase and primase activities of PrimPol variants with separation-of-function point mutations. (**A**) The scheme of PrimPol point mutations and deletions. (**B**) The DNA polymerase activity of PrimPol mutant variants. Reactions were incubated with the $^{32}$P-labeled Template-55/Primer-18 substrate for 2–30 min; pH 7.0 and 10 mM $Mg^{2+}$. Lanes 10–12 contain an equimolar mix of $PrimPol_{1-363}$ and $PrimPol_{363-560}$ proteins, lanes 19–21 contain an equimolar mix of $PrimPol_{D114A}$ and $PrimPol_{R417A}$ proteins; lanes 25–27 contain an equimolar mix of $PrimPol_{D114A}$ and $PrimPol_{R424A}$ proteins. (**C**) The DNA primase activity of PrimPol variants. Reactions were incubated in the presence of [γ-$^{32}$P]-ATP, ATP, dGTP and dTTP (lanes 1–7) or [γ-$^{32}$P]-ATP, ATP and dGTP (lanes 8–14); pH 7.0 and 1 mM $Mn^{2+}$. Lanes 6 and 12 contain an equimolar mix of $PrimPol_{D114A}$ and $PrimPol_{R417A}$ proteins; lanes 7 and 13 contain an equimolar mix of $PrimPol_{D114A}$ and $PrimPol_{R424A}$ proteins.

primase activity in the NTD preparation was verified using the catalytically inactive $PrimPol_{1-363CD}$ variant harboring the D114A substitution (Supplementary Figure S3) and mass spectrometry.

At pH 7.0, $PrimPol_{1–363}$ shows weak primase activity, while $PrimPol_{363–560}$ has no catalytic activity (Figure 3A). When NTD and CTD were mixed together, the primase activity was partially restored (Figure 3A, lanes 5 and 9). This result points to cooperation of the two separated domains during initiation of DNA synthesis and to the possible interaction between them. Indeed, with a broken linkage between the NTD and CTD, only a relatively stable interaction of these two domains could result in almost complete restoration of primase activity. The notion of interaction between the two PrimPol domains upon dinucleotide formation is supported by EMSA with single-stranded DNA (Figure 3B, lanes 14–16). Importantly, $PrimPol_{1-363}$ makes a stable complex with DNA, while the full-length PrimPol and $PrimPol_{363–560}$ do not bind ssDNA. Mixing NTD and CTD together resulted in the loss of $PrimPol_{1–363}$ ability to bind ssDNA. These data suggest that the CTD of PrimPol might prevent efficient binding of the protein to DNA. The high affinity of $PrimPol_{1–363}$ to DNA in the absence of CTD may explain its high DNA polymerase activity (Figure 2B). Thus, CTD demonstrates negative regulation of ssDNA binding by NTD. This is the opposite of human primase, where PriS has very low affinity to DNA and CTD plays the main role in template-primer binding (5). However, CTD did not abolish the NTD binding to the template-55/primer-18 (Figure 3B, lanes 27–29). The absence of a

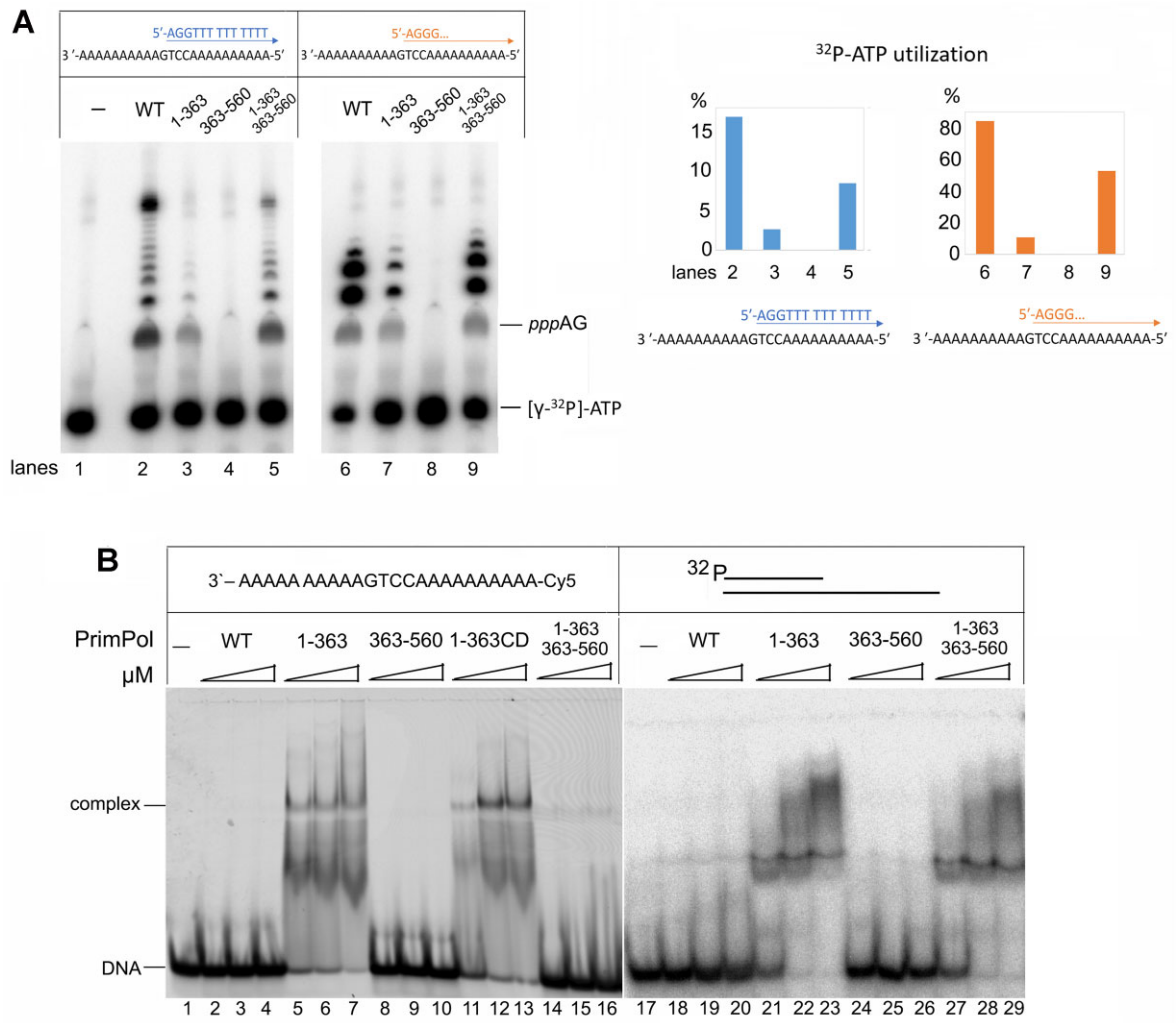


**Figure 3.** The primase activity analysis of PrimPol variants with separate NTD and CTD. (**A**) The DNA primase activity of PrimPol variants. Reactions were incubated in the presence of [γ-$^{32}$P]-ATP, ATP, dGTP and dTTP (lanes 2–5) or [γ-$^{32}$P]-ATP, ATP and dGTP (lanes 6–9); pH 7.0 and 1 mM $Mn^{2+}$. (**B**) EMSA of ssDNA Cy5-Acctg and the $^{32}$P-labeled Template-55/Primer-18 binding by PrimPol variants. Reactions were incubated with 1, 2 or 3 μM PrimPol in the presence of 300 nM DNA and 1 mM $Mn^{2+}$, pH 7.0.

negative effect of CTD on the NTD/dsDNA complex can be explained by the higher PrimPol affinity to a primed DNA template versus ssDNA, which is less structured and can bind to NTD at non-specific sites.

### The ZnFn Arg417 and Arg424 are required for interaction with the 5′-triphosphate

During *de novo* DNA synthesis, PrimPol predominantly uses ATP (or dATP) as the initiator nucleotide, and the ZnFn motif of the CTD plays a key role in binding and incorporation of ATP by an unknown mechanism (17,23). We propose that the conservative ZnFn residues Arg417 and/or Arg424 may play a role in binding the 5′-triphosphate of the initiating ATP, which becomes the first nucleotide of the primer. We selected these arginines because they are the most conservative in the ZnFn motif (7). By analogy, in PriL of human primase, Arg302 and Arg306 interact with the 5′-triphosphate of a primer and play a key role in primase activity (31). Indeed, the R417A and R424A substitutions disrupted the DNA primase activity of PrimPol in this work. To study the possible role of these arginines in the 5′-triphospate binding, a DNA substrate with a primer containing adenosine triphosphate at the 5′-end was synthesized enzymatically using PrimPol (Figure 4A and Supplementary Figure S1).

We have shown that the wild-type and mutant forms of PrimPol cannot efficiently bind the DNA duplex without a triphosphate at the 5′-end (Figure 4B, lanes 2–7). In contrast, PrimPol binds the DNA duplex with the 5′-triphosphate very efficiently (Figure 4B, lanes 8–9). Moreover, the R424A and R417A substitutions dramatically decreased the ability of PrimPol to bind DNA with the 5′-triphosphate (Figure 4B, lanes 10–13, Table 2, and Supplementary Figure S4). These data indicate that the CTD holds the primer 5′-end while the NTD extends the primer 3′-end, which resembles the division of labor between the catalytic and regulatory domains of human primase.

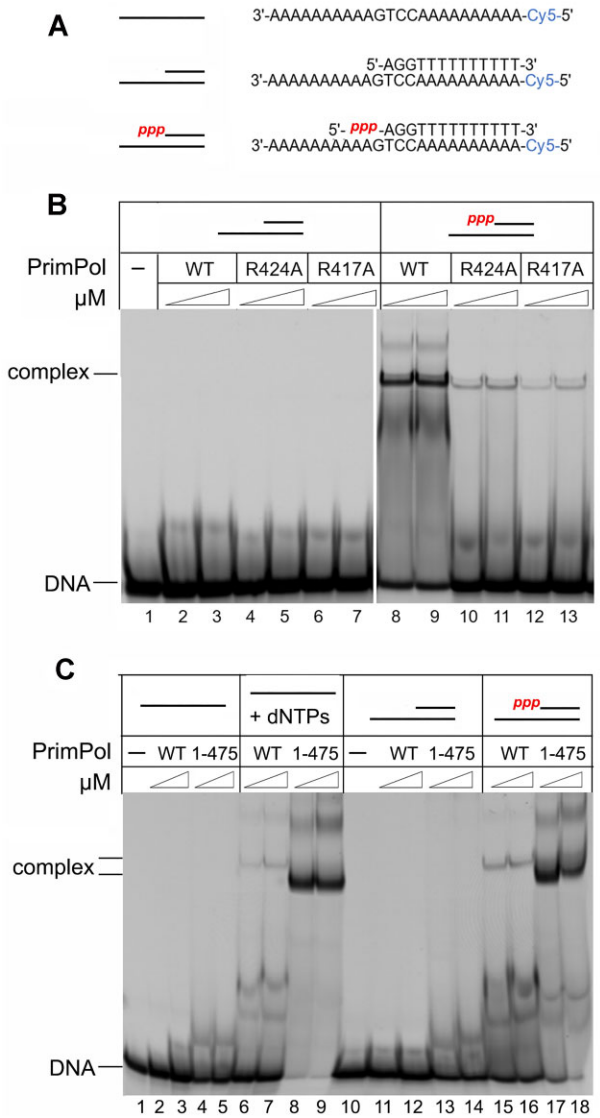


**Figure 4.** The role of 5′-triphospate and RBM in DNA binding of PrimPol. (**A**) Structures of DNA substrates used in the study. (**B**) EMSA of binding to DNA with 5′-triphospate by PrimPol ZnFn mutant variants. Reactions were incubated with 1 or 2 μM PrimPol in the presence of 300 nM DNA and 1 mM $Mn^{2+}$, pH 7.0. (**C**) EMSA of binding to DNA by the PrimPol RBM mutant variant. Reactions were incubated with 1 or 2 μM PrimPol in the presence of 300 nM DNA and 1 mM $Mn^{2+}$, pH 7.0. ATP, dGTP and dTTP were added to some reactions (lanes 6–9).

### The RBM modulates PrimPol binding to DNA

In addition to the ZnFn motif, the CTD contains another important element, the RPA-binding motif (RBM), which is rich in negatively charged a.a. (32). It can be assumed that this motif may affect the affinity of PrimPol to DNA by interacting with RPA. Indeed, the $PrimPol_{1\text{-}475}$ variant with deletion of the RBM showed increased DNA binding efficiency to DNA with the 5′-triphosphate compared to the full-length protein (Figure 4C, lanes 17, 18). These data demonstrate the key role of the CTD and particularly the RBM motif in the regulation of PrimPol binding to DNA. The mechanism of regulation of PrimPol activity may be due to the interaction of RPA or other proteins with the negatively charged RBM and neutralization of the excess charge, which promotes DNA binding by the enzyme and its activity.

**Table 2.** Average binding affinities ($K_D$) of PrimPol variants to DNA with the 5′-triphospate determined by EMSA

| Protein | $K_D$ for '*p*-*p*-*p*-12', μM | $K_D$ for '*p*-*p*-*p*-8', μM |
|---|---|---|
| $PrimPol_{WT}$ | 0.49 ± 0.13 | 0.78 ± 0.12 |
| $PrimPol_{1\text{-}475}$ | 0.09 ± 0.01 | 0.11 ± 0.01 |
| $PrimPol_{R417A}$ | >10* | - |
| $PrimPol_{R424A}$ | >10* | - |

*$K_D$ values for the $PrimPol_{R424A}$ and $PrimPol_{R417A}$ variants are >10 μM. Exact $K_D$ values were not calculated because they are out of range of protein concentrations in the reaction (Supplementary Figure S4).

As expected, DNA binding of the wild-type PrimPol and the $PrimPol_{1–475}$ variant was more efficient on DNA with the 5′-triphosphate, which was synthesized and purified before analysis (Figure 4B, lanes 15-18) or incorporated by PrimPol during *de novo* DNA synthesis directly in EMSA reactions (Figure 4B, lanes 6–9).

To demonstrate the role of the RBM and RPA in the regulation of PrimPol activity, we analyzed PrimPol binding to ssDNA preincubated with RPA (Figure 5A). The full-length PrimPol formed complexes with DNA and RPA:DNA (Figure 5A, lanes 6–8). The $PrimPol_{1–475}$ variant efficiently formed a complex with ssDNA alone but not with RPA:DNA (Figure 5A, lanes 13–15). The deletion of the RBM in $PrimPol_{1–475}$ variant also abrogated the stimulation of PrimPol by RPA in DNA polymerase reactions (Figure 5B, lanes 20–25). The R417A and R424A substitutions in ZnFn did not affect the PrimPol stimulation by RPA (Supplementary Figure S5).

## DISCUSSION

### Human PrimPol initiates DNA synthesis in *cis*-orientation

The relative orientation of PrimPol domains during *de novo* DNA synthesis is important for understanding the topology of the replication fork and its regulation by other proteins. Previously, the *cis*/trans mode of action was studied for several DNA primases (24–26,33). In this work, we demonstrated that the full-length PrimPol operates in *cis*-orientation when the catalytic NTD and regulatory CTD of the same molecule are involved in *de novo* DNA synthesis. This mechanism is similar to that previously described for replicative human primase PriS/PriL (5). On the other hand, experiments with separated NTD and CTD revealed their ability to cooperate during DNA synthesis initiation. We assume that the actual test for the *trans*-mechanism of DNA synthesis priming is an experiment involving point mutations (when a molecule with a mutation in the CTD can complement primase activity of the NTD mutant). Experiments with CTD and NTD mixed together revealed their ability to cooperate without the covalent link between them, which points to non-covalent complex formation. The interaction between the catalytic and regulatory domains would stabilize the initiation complex composed of

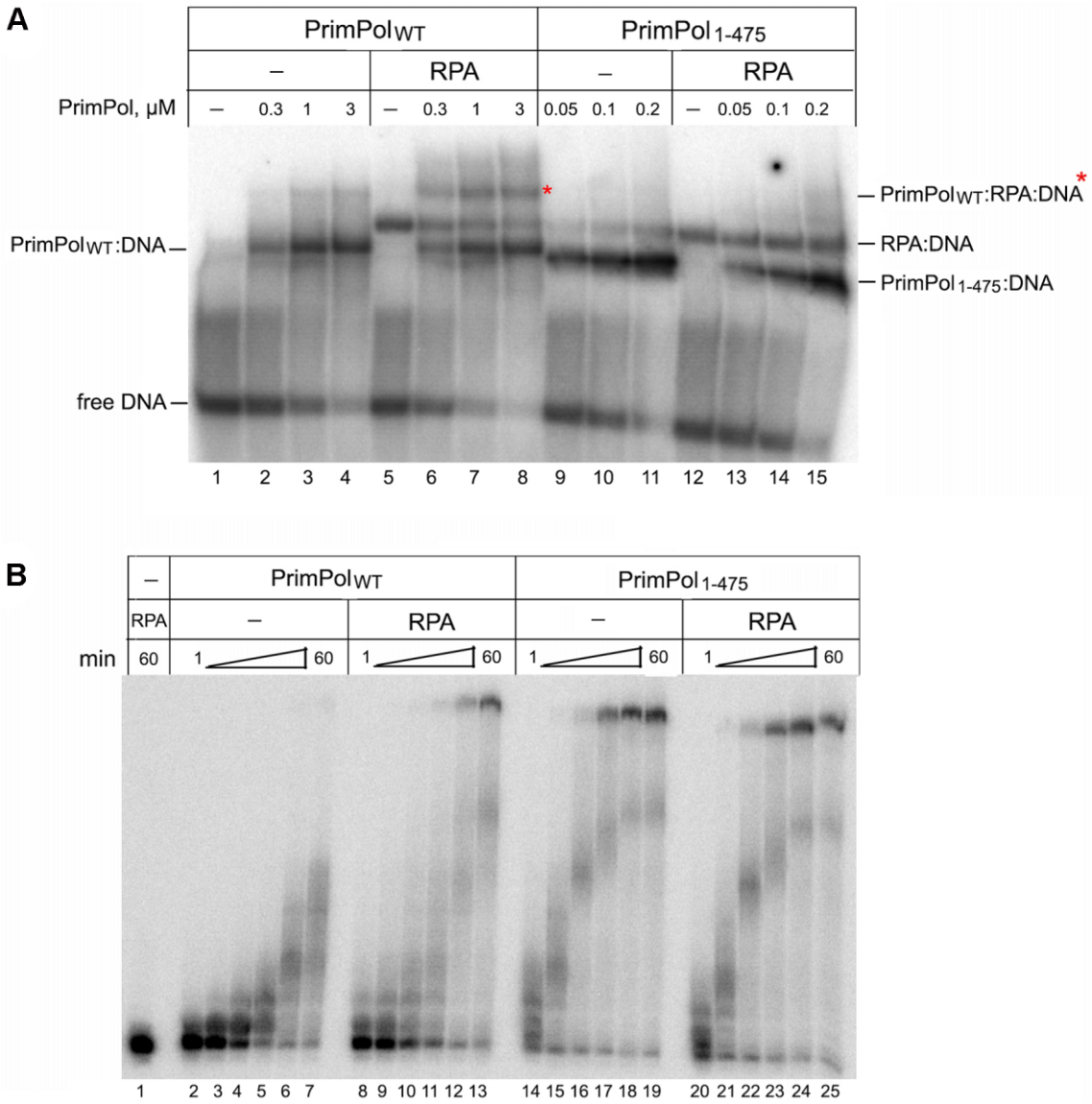


**Figure 5.** The regulation of PrimPol by RPA. (**A**) EMSA of PrimPol binding to ssDNA and ssDNA/RPA. Reactions were incubated with 0.3–3 μM PrimPol or 0.05–0.2 μM $PrimPol_{1-475}$ variant in the presence of 50 nM $^{32}$P-labeled Acctg55 ssDNA, 25 nM RPA (lanes 5–8 and 12–15), 1 mM ATP, 200 μM dNTPs, 1 mM $Mn^{2+}$, at pH 7.0 and 8°C. (**B**) The stimulation of the DNA polymerase activity of PrimPol and $PrimPol_{1-475}$ by RPA. Reactions were started by addition of 100 nM PrimPol and incubated for 1–60 min; pH 7.0 and 10 mM $Mg^{2+}$. When applicable, the $^{32}$P-labeled Template-55/Primer-18 substrate was preincubated with 10 nM RPA at 30°C for 3 min.

several weakly bound components: PrimPol, DNA template, and two NTPs.

The *cis*-mechanism suggests that the CTD bound to the primer 5′-end limits the length of a newly synthesized primer, resulting in DNA synthesis termination due to inability of the NTD to extend the primer 3′-end. Indeed, it was demonstrated that PrimPol pauses after synthesis of a ∼ 10-mer primer (3). According to the model of ZnFn/T:P complex, the length of a DNA duplex should not affect ZnFn interaction with a template-primer. The reason for synthesis termination could be a clash between the NTD and CTD as was shown for human primase (5) or the tension in the inter-domain linker as was shown for human primosome (34). In the course of primer synthesis, the distance between the NTD and CTD will increase and they will rotate relative each other making one turn when the primer length is 10–11 nucleotides. To relax the twisted linker, one of PrimPol domains should temporarily dissociate from the template-primer. The mechanism of primer synthesis pausing is a subject of future research.

### The role of the ZnFn motif in primase activity

The C-terminal ZnFn motif plays a key role in the primase activity of PrimPol (17,23). In this work, we demonstrated that the triphosphate at the 5′ end of the primer is required for efficient binding of PrimPol to the template-primer. Our results support the findings of Martínez-Jiménez *et al.* (17)

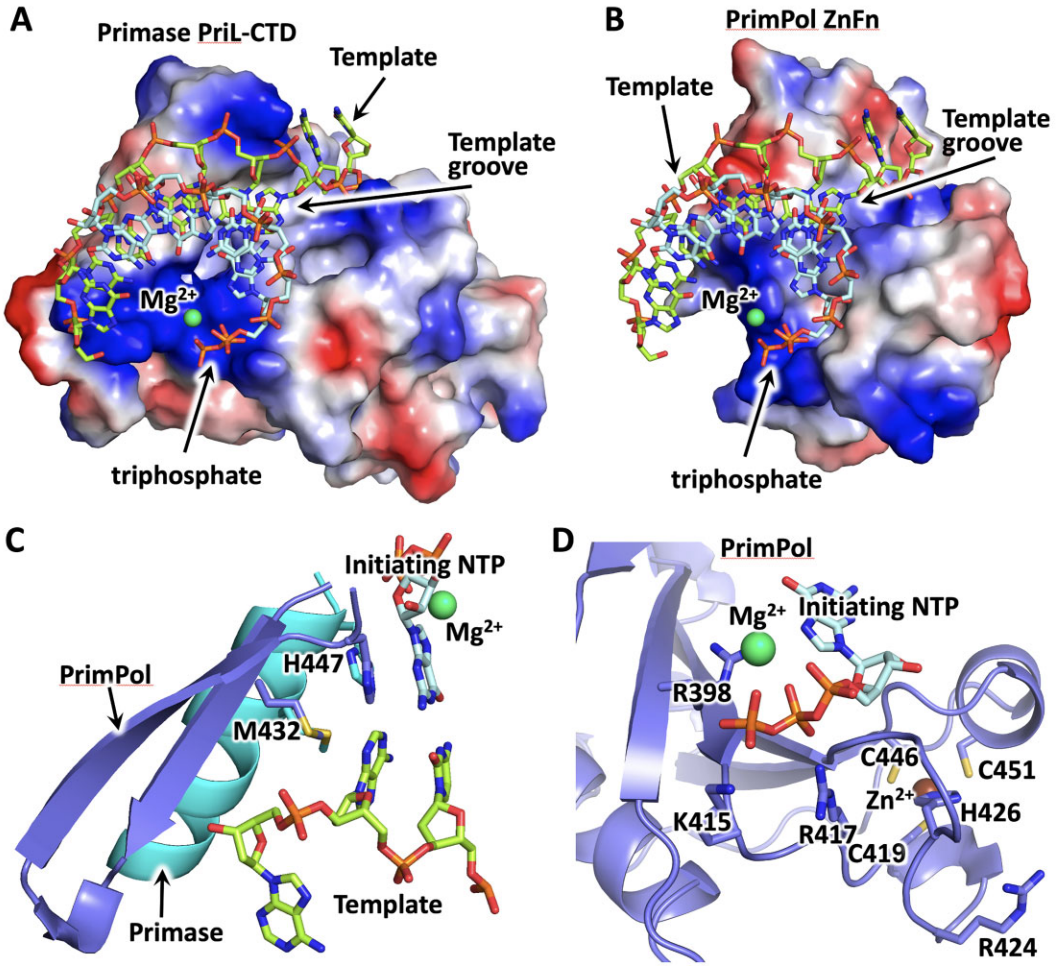


**Figure 6.** ZnFn and PriL-CTD bind the template-primer and the initiating nucleotide in a similar mode. Comparison of template-primer binding by PriL-CTD (**A**) and ZnFn (**B**) using the coordinates of the PriL-CTD/DNA:RNA complex (PDB ID 5F0Q) and human PrimPol obtained from the AlphaFold database (accession code AFQ96LW4). The protein surface is represented by the vacuum electrostatic potential. (**C**) Met432 and His447 of PrimPol stabilize the initiating base-pair and the template base preceding it. The corresponding residues of PriL-CTD have the same position. (**D**) The close-up view of the initiation site of PrimPol. DNA, RNA, and amino acids are shown as sticks. PriL-CTD and PrimPol residues colored cyan and slate, respectively. $Mg^{2+}$ and $Zn^{2+}$ ions are shown as spheres and colored green and brown, respectively. The figure was prepared using the PyMOL Molecular Graphics System (version 1.8, Schrödinger, LLC).

showing that PrimPol uses nucleotide triphosphate as the initiating nucleotide (di- and monophosphates significantly reduce primase activity).

Despite the key role of the ZnFn in primase activity, the NTD of PrimPol lacking the ZnFn retains weak primase activity. These data are in agreement with recent studies showing weak primase activity of the NTD of PrimPol (deletion 410–560 a.a.) (17) and CRISPR-associated primase-polymerases (35) in reaction with $Mn^{2+}$. In an earlier study, the mutant N-terminal variant $PrimPol_{1–354}$ completely lacked primase activity (12). Contrasting results may be related to sensitivity of the NTD activity to the low pH (7.0) widely used in assays and to the absence of $Mn^{2+}$ ions in primase reaction. In addition, one NTD molecule may assist another by binding the initiating nucleotide during dinucleotide synthesis. Higher primase activity of NTD at pH 7.5 can be explained by more stable/optimal interaction between the two NTD molecules during dinucleotide formation.

We built the model of the ZnFn/template-primer complex using the coordinates of human PrimPol obtained from the AlphaFold database (accession code AFQ96LW4) and assuming that Arg417 interacts with a 5′-triphosphate of a primer (Figure 6). The template-primer obtained from the PriL-CTD/DNA–RNA structure (pdb ID 5f0q) was manually fitted into the model using 'align to molecule' function in PyMOL, by placing the DNA template into the positively charged groove and the triphosphate close to Arg417. Strikingly, the model revealed high similarity in structural organization of the initiation site in the ZnFn and PriL-CTD, including the histidine and methionine residues that stabilize the template base preceding the initiating base-pair (Figure 6C) and two arginines interacting with a triphosphate (Figure 6D). Thus, despite significant difference in the overall fold and coordinated metals (PriL coordinates the 4Fe-4S cluster), ZnFn and PriL-CTD bind the initiating nucleotide and the template-primer in a similar way. According to the model, Arg424 cannot interact with a 5′-triphosphate

(Figure 6D), which is consistent with the weaker effect of its mutation on primase activity in comparison to Arg417 (Figure 2C). Arg424 may play a structural or the other role in DNA synthesis priming; for example, in CTD interaction with NTD in the initiation complex. Of note, the initiation site of PrimPol is located in close proximity to the zinc-binding site.

### The role of the RBM in primase activity

We demonstrated that the CTD negatively regulates the DNA polymerase activity of full-length PrimPol. Moreover, the RBM of the CTD is involved in modulation of PrimPol binding to DNA with the 5′-triphosphate. PrimPol co-purifies from human cells along with RPA and mtSSB (3). RPA recruits PrimPol to DNA damage sites in cells, and RBM deletion increases cell sensitivity to DNA damaging agents (3). In addition, RPA stimulates the DNA polymerase and DNA primase activities of PrimPol *in vitro* (32,36). Our data suggest that RPA counteracts the negative regulatory effect of the CTD on DNA binding. PrimPol itself is auto-inhibited ('off' state) and becomes fully functional upon RPA binding ('on' state).

PrimPol binds to the NTD of the RPA1 subunit (RPA70N) (20,32). On the surface of RPA70N, there is a positively charged region responsible for interaction with many RPA partner proteins (37). The RBM-B motif, on the contrary, contains negatively charged amino acids whose mutations (residues Asn551 and Glu548) disrupt the interaction of PrimPol with RPA. The possible mechanism of PrimPol regulation by RPA may include an increase in affinity to DNA as a result of structural reorganization of the CTD favoring its interaction with a template and initiating ATP. For example, RPA may prevent RBM from blocking the DNA-binding site of ZnFn. Neutralization of the negative charge of RBM upon RPA binding can promote PrimPol-DNA interaction as well.

A separate CTD only slightly inhibits the DNA polymerase activity of the NTD, which is consistent with almost no effect of CTD on template-primer binding by NTD. It can be assumed that the DNA polymerase activity of PrimPol on a DNA template with a primer lacking the 5′-triphosphate mainly depends on the DNA-binding properties of the NTD. Therefore, the inhibitory effect of CTD is stronger than its stimulatory effect based on CTD interaction with a 5′-triphosphate.

## CONCLUSIONS

Similar to human DNA primase, the results of this study indicate a division of work between the NTD and CTD of human PrimPol: the NTD is responsible for catalysis, and the CTD holds the template-primer. The *cis*-orientation during *de novo* DNA synthesis, the regulatory role of the CTD in DNA binding, and the requirement of a triphosphate group for stabilization of initiation and elongation complexes of PrimPol with DNA together describe the mechanism of primase activity similar to that seen in human primase.

## DATA AVAILABILITY

The data that support the findings of this study are included in the Supplementary Data file or available from the corresponding author upon request.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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