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The invariant glutamate of human PrimPol DxE motif is critical for its Mn^{2+}-dependent distinctive activities

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ABSTRACT

PrimPol is a human primase/polymerase specialized in downstream repriming of stalled forks during both nuclear and mitochondrial DNA replication. Like most primases and polymerases, PrimPol requires divalent metal cations, as Mg^{2+} or Mn^{2+}, used as cofactors for catalysis. However, little is known about the consequences of using these two metal cofactors in combination, which would be the most physiological scenario during PrimPol-mediated reactions, and the individual contribution of the putative carboxylate residues (Asp^{114}, Glu^{116} and Asp^{280}) acting as metal ligands. By site-directed mutagenesis in human PrimPol, we confirmed the catalytic relevance of these three carboxylates, and identified Glu^{116} as a relevant enhancer of distinctive PrimPol reactions, which are highly dependent on Mn^{2+}. Herein, we evidenced that PrimPol Glu^{116} contributes to error-prone tolerance of 8oxodG more markedly when both Mg^{2+} and Mn^{2+} ions are present. Moreover, Glu^{116} was important for TLS events mediated by primer/template realignments, and crucial to achieving an optimal primase activity, processes in which Mn^{2+} is largely preferred. EMSA analysis of PrimPol:ssDNA:dNTP pre-ternary complex indicated a critical role of each metal ligand, and a significant impairment when Glu^{116} was changed to a more conventional aspartate. These data suggest that PrimPol active site requires a specific motif A (DxE) to favor the use of Mn^{2+} ions in order to achieve optimal incoming nucleotide stabilization, especially required during primer synthesis.

1. Introduction

In silico analyses of the primary sequence of human PrimPol indicate that it belongs to the archaeo-eukaryotic superfamily of primases (AEP) containing a diverged version of the RNA recognition motif (RRM) fold [1]. The AEP superfamily is highly heterogeneous as it encompasses conventional primases, PrimPols, and even RNA polymerases specialized in the nonhomologous end-joining (NHEJ) of double-strand breaks (DSBs) [2,3]. PrimPol harbors the conserved A, B, C motifs of the AEP superfamily and also a C-terminal subdomain containing a Zinc-finger motif, similar to that of herpesvirus UL52 primase and other AEP-like enzymes, which was shown to be essential for its primase activity [4–6].

PrimPol is the first DNA primase characterized in human cells, as it is able to start DNA chains with deoxynucleotides, with a unique active site for both DNA primase and DNA polymerase activities [7–9]. Human PrimPol provides different trans-lesion synthesis (TLS) alternatives when DNA replication is stalled: 1) PrimPol can synthesize DNA primers ahead of lesions, such as UV lesions and G-quadruplexes, to allow DNA replication to continue [4,10]; 2) PrimPol can realign primers ahead of "unreadable lesions" such as abasic sites and pyrimidine(6-4) pyrimidone photoproducts (6-4PPs), thereby skipping the lesion [11]; 3) PrimPol can incorporate nucleotides opposite DNA lesions such as 8oxodG, like a regular TLS DNA polymerase [7,11,12]. Cellular fractionation experiments have shown that PrimPol localizes to both the nucleus and mitochondria of human cells providing a new and more ergonomic solution for priming: the direct synthesis of DNA primers [4,7]. PrimPol primase activity was demonstrated to be relevant for reinitiating stalled forks after UV damage during nuclear DNA replication [4]. Besides, PrimPol primase activity was shown to be also required during mitochondrial DNA replication, by reinitiating synthesis after UV damage or in the presence of chain-terminating nucleotides [7,13].

Primase and DNA polymerases, despite the high degree of

Abbreviation: AEP, archaeo-eukaryotic primase

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specialization, have common characteristics including a two-metal-ion mechanism of catalysis [14]. Metal ion A establishes interactions with the OH group of the 3′-end of the nascent DNA chain. This interaction facilitates the attack of the hydroxyl group on the α-phosphate of the incoming dNTP [15]. Both metal ions help to stabilize the intermediate that occurs during phosphodiester bond formation. Catalysis occurs through a chemical reaction type SN2 where the α phosphate goes through a transition state that involves a pentacovalent phosphate intermediate. In the final step of the reaction, metal ion B facilitates the release of the pyrophosphate during phosphodiester bond formation [16]. Recent work reveals a possible intervention of a third metal in catalysis, probably stabilizing the transition state and facilitating the release of the product [17–20]; however, the role of the third metal ion remains an active area of debate, as it has been recently proposed that the third metal stabilizes the formed pyrophosphate product, rather than help to catalyze the reaction [21].

Primases and DNA polymerases often require three carboxylates within the active site to coordinate the two metal cofactors and form a metal bridge between the two reacting substrates, the primer terminus and the incoming nucleotide [16,22]. These metal ligands are generally aspartates and two of them are localized close together forming what is called a DxD motif (motif A) in AEP primases. Eukaryotic PrimPols, including human PrimPol, have a slightly different motif since the second carboxylate is not an aspartate residue, but a glutamate residue [1]. The DxE variant is also present in PrimPol of plasmid pRN1 from *Sulfolobus islandicus,* which has DNA primase-polymerase activity [23].

Despite Mn^{2+} decreases the fidelity of human primase, it also stimulates its activity by increasing the rate of both initiation and elongation in a template-dependent manner [24]. Furthermore, increasing evidence indicates that X and Y-family DNA polymerases including Pol ι, Pol β, Pol μ, and Pol λ stimulate their catalytic activity by physiological concentrations of Mn^{2+} ions [25–28]. PrimPol can catalyze DNA synthesis using either Mg^{2+} or Mn^{2+}, although it shows a clear preference for the latter as it stimulates its overall activity [7,11]. Recent studies demonstrated that in contrast to Mn^{2+}, Mg^{2+} favors error-free bypass of 8oxoG by PrimPol, although it decreases PrimPol overall activity [12,29]. Interestingly, even though Mg^{2+} and Mn^{2+} are known to be simultaneously present in the cell, the effect of the combination of both metal cofactors on PrimPol-mediated reactions has never been considered. Here we compared the enzymatic unusual properties of human PrimPol in the individual or combined presence of both Mg^{2+} and Mn^{2+} activating metal ions. Whereas Mg^{2+} favors PrimPol fidelity during 8oxoG bypass, Mn^{2+} boosts PrimPol overall activity being indispensable for stabilizing the incoming nucleotide, which is especially required during primase and primer/template dislocation events. In this work, we studied the individual contribution of the putative carboxylate residues (Asp^{114}, Glu^{116} and Asp^{280}) acting as metal ligands in human PrimPol, and showed that PrimPol active site might be adapted to use Mn^{2+} by virtue of Glu^{116}, the distinctive amino acid at the DxE motif, which stabilizes the incoming nucleotide at the 3′-site during Mn^{2+}-dependent PrimPol activities.

2. Materials and methods

2.1. Protein, oligonucleotides and nucleotides

Human PrimPol and single point mutants E116D, D114 A, E116 A, D280 A were constructed (by site-directed mutagenesis), expressed and purified as previously described [4,7].

Conventional DNA oligonucleotides were synthesized by Sigma Aldrich (St Louis, MO, USA). Oligonucleotides used in the in vitro DNA polymerization assays were purified by 8 M urea-20% polyacrylamide gel electrophoresis. Oligonucleotides used as primers in those assays were 5′ labeled with [γ-^{32}P]*ATP* (3000 Ci/ mmol) from Perkin-Elmer (Waltham, MA, USA) and the T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) for 45 min at 37 °C. Hybridizations were performed in 1X hybridization buffer (50 mM Tris-HCl pH 7.5 /0.3 M NaCl) for 10 min at 80 °C. Ultrapure dNTPs were supplied by GE (Fairfield, CT, USA). Radiolabeled nucleotides [γ-^{32}P]*ATP*, [α-^{32}P] dGTP (3000 Ci/mmol,) were purchased from Perkin Elmer (Waltham, MA, USA).

2.2. Primary sequence alignments

Multiple alignments of the indicated amino acid sequence were performed using the MULTALIN server [30](http://multalin.toulouse.inra.fr/multalin/). Three-dimensional images and modeling of mutated residues were created with Swiss-PdbViewer (DeepView) program, developed within the Swish Institute of Bioinformatics (SIB) at the Structural Bioinformatics Group at the Biozentrum in Basel, and using *Hs*PrimPol PDB ID: 5L2X, which corresponds to the crystal structure of human PrimPol ternary complex [31].

2.3. DNA polymerization assays

For primer extension characterization of metal ligands residues, a 5′ [^{32}P]-labeled 15-mer DNA primer (5′-GATCACAGTGAGTAC-3′) was hybridized to a 28-mer DNA undamaged template (5′-AGAAGTGTATC TAGTACTCACTGTGATC-3′) incubated with 200 nM of either WT PrimPol or mutants E116D, D114 A, E116 A, D280 A providing dNTPs (1, 10, 100 μM) in the presence of 1 mM $MnCl_2$. The reaction mixture (20 μL) contained reaction buffer (50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol (DTT), 4% glycerol, 0.1 mg/ml bovine serum albumin (BSA) and 2.5 nM of the template/primer DNA. After 30 min of incubation at 30 °C reactions were stopped by addition of formamide buffer (95% (v/v) formamide, 10 mM EDTA, 0.1% (w/v) xylene cyanol and 0.1% (w/v) bromophenol blue) and resolved by 8 M urea-20% polyacrylamide gel electrophoresis.

For “standing start” analysis of 8oxodG tolerance, a 5′ [^{32}P]-labeled 15-mer DNA primer (5′- CTGCAGCTGATGCGC-3′) was hybridized to a 34-mer DNA template (5′- GTACCCGGGGATCCGTAC8GCGCATCAGCT GCAG-3′), and incubated with purified PrimPol WT or E116D mutant (200 nM) in the presence of 10 μM of either dATP or dCTP and the indicated concentrations metal cofactors $MgCl_2$ or $MnCl_2$. The reaction mixture (20 μL) contained reaction buffer and 2.5 nM of the template/primer DNA. After 30 min of incubation at 30 °C, primer extension was analyzed as described previously and band intensities were quantified by densitometry.

To evaluate the PrimPol capacity to induce distortions in the template or primer, a 5′ [^{32}P]-labeled 15-mer DNA primer (5′-GATCACA GTGAGATC-3′) was hybridized to 5′-AGAAGTGTATCTCGTACTCACTG TGATC-3′. Assays were performed in reaction buffer 1 mM $MnCl_2$, 2.5 nM of the DNA/primer, 200 nM PrimPol WT or E116D variant and the indicated type and concentration of deoxynucleotides. After 30 min of incubation at 30 °C, primer extension was analyzed as described previously.

For synthesis fidelity analysis a 5′ [^{32}P]-labeled 15-mer DNA primer (5′- CTGCAGCTGATGCGC-3′) was hybridized to a 34-mer DNA undamaged template (5′- GTACCCGGGGATCCGTACGGCGCATCAGCTG CAG-3′), and incubated with purified PrimPol wild-type or E116D mutant (200 nM) in the presence of each individual dNTP (10 μM) in the presence of 1 mM $MnCl_2$ or 5 mM $MgCl_2$. After 30 min of incubation at 30 °C, primer extension was analyzed as described previously.

PrimPol primer realignment capacity was evaluated by incubating purified PrimPol WT or E116D variant (200 nM) with a 5′ [^{32}P]-labeled 14-mer DNA primer (5′- CACTGACTGTATGATG-3′) hybridized to a 30-mer DNA template (5′- CTCGTCAGCATGTTCATCATACAGTCAGTG-3′). The reaction mixture contained 2.5 nM of the labeled DNA, 10 μM of either dATP or dCTP and the indicated concentrations of Mn^{2+} or Mg^{2+}. After 30 min of incubation at 30 °C, primer extension was analyzed as described previously.

2.4. DNA primase assay

DNA primase activity of WT PrimPol and E116D, D114 A, E116 A, D280 A mutants was evaluated using a 29-mer oligonucleotide (5′-T_{15}CCTGT$_{10}$-3′) as template [7]. The reaction mixture (20 μL) in buffer R (50 mM Tris pH 7.5, 5% Gly, 75 mM NaCl, 1 mM $MnCl_2$, 1 mM DTT and 0.1 μg/μL BSA), contained PrimPol (400 nM), [γ-^{32}P]ATP (16 nM) and increasing concentrations of dGTP (1, 10 and 100 μM). The reaction was incubated during 30 min at 30 °C. Dimer formation by WT PrimPol or E116D variant (400 nM) on 3′GTCA5′ (1 μM) sequence providing dGTP (1 μM) and [γ-^{32}P]*ATP* (16 nM) in the presence of $MnCl_2$ (0.1, 0.5, 1 mM); $MgCl_2$ (5 mM) or $MnCl_2$ (0.1, 0.5, 1 mM) + $MgCl_2$ (5 mM). Reactions were stopped by addition of formamide buffer and loaded in 8 M urea–containing 20% polyacrylamide sequencing gels. To obtain velocities, quantification of dimer formation by WT PrimPol or E116D variant (400 nM) on 3′GTCA5′ (1 μM) sequence, in the presence of 1 mM $MnCl_2$ providing dGTP (1 μM) and [γ-^{32}P]*ATP* (16 nM) at different times of reaction (0, 60, 120, 240, 360, 480 s).

2.5. EMSA for enzyme: ssDNA binary complex

Enzyme:ssDNA binding was performed in buffer A [50 mM Tris-HCl pH 7.5, 40 mM NaCl, 2.5% (w/v) glycerol, 2.5% (w/v) PEG-4000, 1 mM DTT, 0.1 mg/ml BSA], using PNK-[γ-^{32}P]-labeled oligonucleotide 1 nM 3′T_{20}GTCCT$_{36}$5′ and increasing concentrations of either WT PrimPol or E116D, D114 A, E116 A, D280 A mutants (10, 20, 40, 80 nM) in the absence of metal. The reaction (in 20 μL) was incubated at 30 °C for 10 min. Subsequently, loading buffer (30% glycerol, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) was added and the reaction was analyzed in a native 6% polyacrylamide gel run at 150 V for 120 min at 4 °C in Tris-glycine pH 8.3 buffer. After electrophoresis, the gel was dried and the mobility shift of free ssDNA *vs* enzyme:ssDNA complex was analyzed by autoradiography.

2.6. EMSA for enzyme:ssDNA:dNTP preternary complex

Pre-ternary complex formation of WT PrimPol or E116D, D114 A, E116 A, D280 A mutants (1 μM) and labeled nucleotide [α-^{32}P]dGTP was evaluated in buffer B [50 mM Tris pH 7.5, 2.5% (w/v) glycerol, 40 mM NaCl, 1 mM $MnCl_2$, 1 mM DTT and 0.1 μg/μL BSA], supplemented when indicated with ssDNA 3′T_{20}GTCCT$_{36}$5′ (0.5 μM; 60-mer), $MnCl_2$ (1 mM) and/or $MgCl_2$ (5 mM). Reactions (20 μL final volume) were incubated for 10 min at 30 °C. Then loading buffer was added and the reactions were analyzed as described above.

3. Results

3.1. Metal ligands of eukaryotic PrimPols involved in polymerization

Archaeo-eukaryotic primases (AEP) show a highly conserved motif DxD (motif A), involved in metal cofactor coordination [1]. Interestingly, the DxD motif is present in most members of the AEP superfamily including the conventional human primase Prim1, viral and bacterial and phage primases, and also in bacterial AEPs involved in NHEJ, as *Mt*PolDom (Fig. 1A). However some archaeal and most eukaryotic PrimPols have a variant DxE motif, as it has been described in PrimPol from pRN1 [32] and human PrimPol [7](Fig. 1A). The human PrimPol catalytically-dead double mutant AxA (D114 A/E116 A) proved the importance of residues Asp^{114}, Glu^{116} of DxE motif in both primase and polymerase activities, supporting the existence of a common active site [7,9]. Glu^{116}, the distinctive amino acid of DxE motif in human PrimPol, is highly conserved among lower and higher eukaryotic orthologs, suggesting its functional relevance (Fig. 1B). Interestingly, two identified prokaryotic PrimPols, *Bacillus cereus* MCM (a member of the PrimPol family with helicase but also primase-polymerase activities) and *Thermus thermophilus* PrimPol are endowed with the conventional DxD motif (Fig. 1A). Notably, both *Bc*MCM and *Tth*PrimPol can operate more proficiently than human PrimPol using Mg^{2+} [33,34], which could suggest that human PrimPol distinctive Glu^{116} (DxE) could favor the use of Mn^{2+} ions. As expected, individual mutation to alanine of each putative metal ligand of human PrimPol (D114 A, E116 A, D280 A), completely ablated Mn^{2+}-dependent PrimPol primer extension activity (Fig. 2). However, mutation of Glu^{116} to the more conventional metal ligand aspartate (E116D) only slightly reduced primer extension activity (Fig. 2). Subsequently, the E116D variant was used to study the precise role of Glu^{116} during more specialized PrimPol activities.

3.2. Human PrimPol Glu^{116} stimulates dATP incorporation opposite 8oxodG

Previous studies demonstrated that PrimPol copies 8oxodG very efficiently, in fact as efficiently as a regular dG, although it prefers to incorporate dATP slightly over (1,5-fold) the error-free dCTP [7]. Those studies were conducted using Mn^{2+} (1 mM) as metal cofactor; however, a more recent work has demonstrated that Mg^{2+} (10 mM) favors dCTP incorporation (5,7-fold over dATP) opposite 8oxodG by PrimPol, thus increasing its accuracy during 8oxodG TLS [12]. Such a difference prompted us to study the effect of the metal concentration, and also the combination of Mn^{2+} and Mg^{2+} in PrimPol-mediated bypass of 8oxodG, which would be the most physiological scenario. As shown in Fig. 3, Mg^{2+} alone allowed a lower (3-fold) primer extension by PrimPol, compared to Mn^{2+}, at all concentrations tested. In agreement with previous conclusions [12], Mg^{2+} favored dCTP incorporation over dATP at all concentrations tested (Fig. 3A). At low and more physiological Mn^{2+} concentration (below 100 μM), PrimPol was able to incorporate both nucleotides to similar extents, and clearly above the levels obtained at the optimal Mg^{2+} concentration (Fig. 3B). However, as previously described [7] the use of higher Mn^{2+} concentrations, close to 250 μM, promoted the preferential incorporation of dATP over dCTP (Fig. 3B). Next, to test the effect of the combination of both metal cofactors, we performed a similar experiment using variable Mn^{2+} concentrations and a fixed and physiological Mg^{2+} concentration (5 mM). Under these conditions, PrimPol incorporated dCTP with higher efficiency than dATP at Mn^{2+} concentrations below 50 μM (Fig. 3C), resembling the results obtained when Mg^{2+} was provided alone (Fig. 3A). Conversely, when Mn^{2+} concentration was higher than 50 μM, dATP incorporation opposite 8oxodG by PrimPol was more efficient than dCTP insertion (Fig. 3C).

Given the effect that the two alternative metal cofactors had on the efficiency and fidelity of nucleotide incorporation opposite 8oxodG by PrimPol, we sought to determine the possible involvement of the distinctive Glu^{116} residue on metal cofactor selection and on PrimPol-mediated TLS of 8oxodG. Interestingly, the E116D mutation did not affect dCTP incorporation at any Mn^{2+} concentrations tested compared to wild-type (WT) PrimPol, and modestly reduced dATP incorporation (Fig. S1 A). Interestingly, in the presence of Mg^{2+}, the E116D variant incorporated dCTP as WT PrimPol, but the mutation caused a significant decrease in dATP incorporation at 0.25, 0.5 and 1 mM Mg^{2+} (Fig. S1B). Consistently, when variable Mn^{2+} concentrations were combined with 5 mM Mg^{2+}, the E116D mutant significantly decreased the dATP incorporation at all conditions tested, again apparently not altering dCTP incorporation (Fig. 3D, S1C).

Altogether these results suggest that PrimPol Glu^{116} residue directly contributes to the error-prone tolerance of 8oxodG, more markedly when both Mg^{2+} and Mn^{2+} ions were present (Figs. 3D, S1C). Notably, given that dATP incorporation opposite 8oxodG by PrimPol relies more on the presence of Mn^{2+} (Fig. 3), it is tempting to speculate that Glu^{116} residue is necessary for optimal Mn^{2+} coordination.

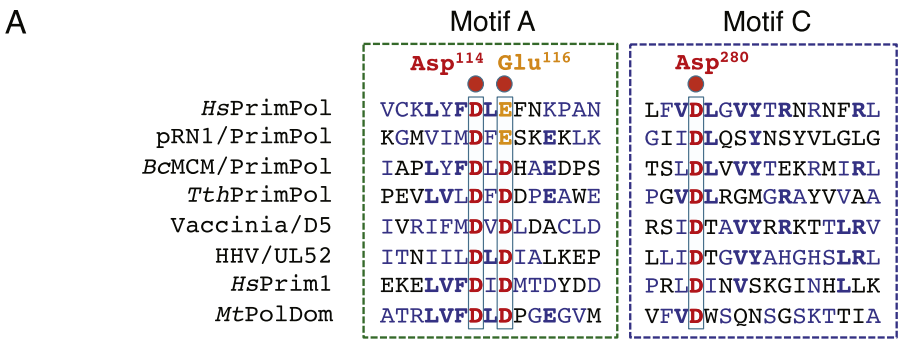


Fig. 1. The DxE metal ligand motif is distinctive of eukaryotic/archaeal PrimPols and highly conserved through evolution. (A) Amino acid sequence alignment of the conserved active-site motif A and motif C of several AEP family members including: *Hs*PrimPol (human PrimPol), pRN1/PrimPol (plasmid pRN1 ORF904 from *Sulfolobus islandicus*), *Bc*MCM/PrimPol (helicase-primase from *Bacillus cereus*), *Tth*PrimPol (*Thermus thermophilus* PrimPol), Vaccinia/D5 (vaccinia virus D5 primase), HHV/UL52 (herpes virus UL52 primase), *Hs*Prim1 (catalytic subunit of the human RNA primase) and *Mt*PolDom (LigD polymerase domain from *Mycobacterium tuberculosis*). Red arrows on the top indicate the metal binding residues (Asp^{114}, Glu^{116}, Asp^{280} of human PrimPol). (B) Multiple amino acid sequence alignment of PrimPol orthologs showing that the DxE metal ligand motif of human PrimPol is highly conserved through evolution. Residues that are either identical or conserved to those in human PrimPol are indicated in red or blue letters, respectively.

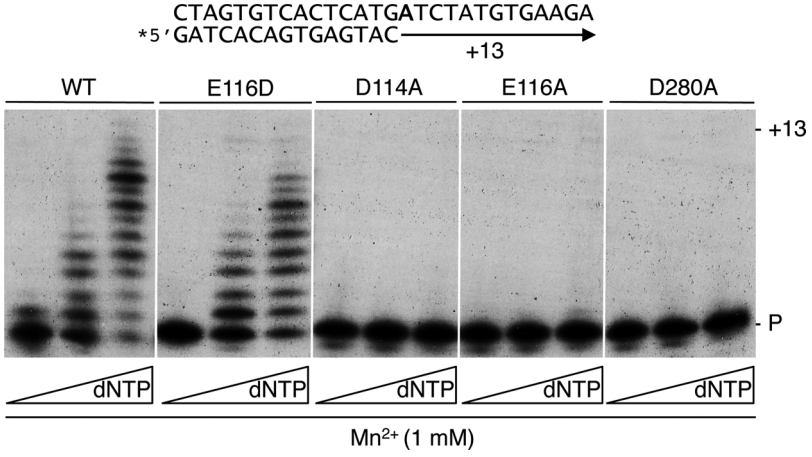


Fig. 2. Metal ligands of human PrimPol are involved in polymerization. Polymerization assay using 2.5 nM of a template/primer structure, 200 nM of either WT PrimPol or mutants E116D, D114 A, E116 A, D280 A providing dNTPs (1, 10, 100 μM) in the presence of 1 mM $MnCl_2$.

3.3. Human PrimPol Glu^{116} enhances Mn^{2+}-dependent dislocation reactions

PrimPol displays a high degree of versatility to accept or induce distortions of both primer and template strands in the vicinity of the active site, creating diverse options of alignment based on DNA sequence microhomology [7,11]. Such a promiscuity of human PrimPol, which confers an alternative solution to skip unreadable template lesions, is strengthened by the use of manganese ions as metal activator [11]. We wonder if Glu^{116} residue could be implicated in these Mn^{2+}-dependent dislocation reactions performed by human PrimPol; hence we compared the WT PrimPol and E116D mutant in different

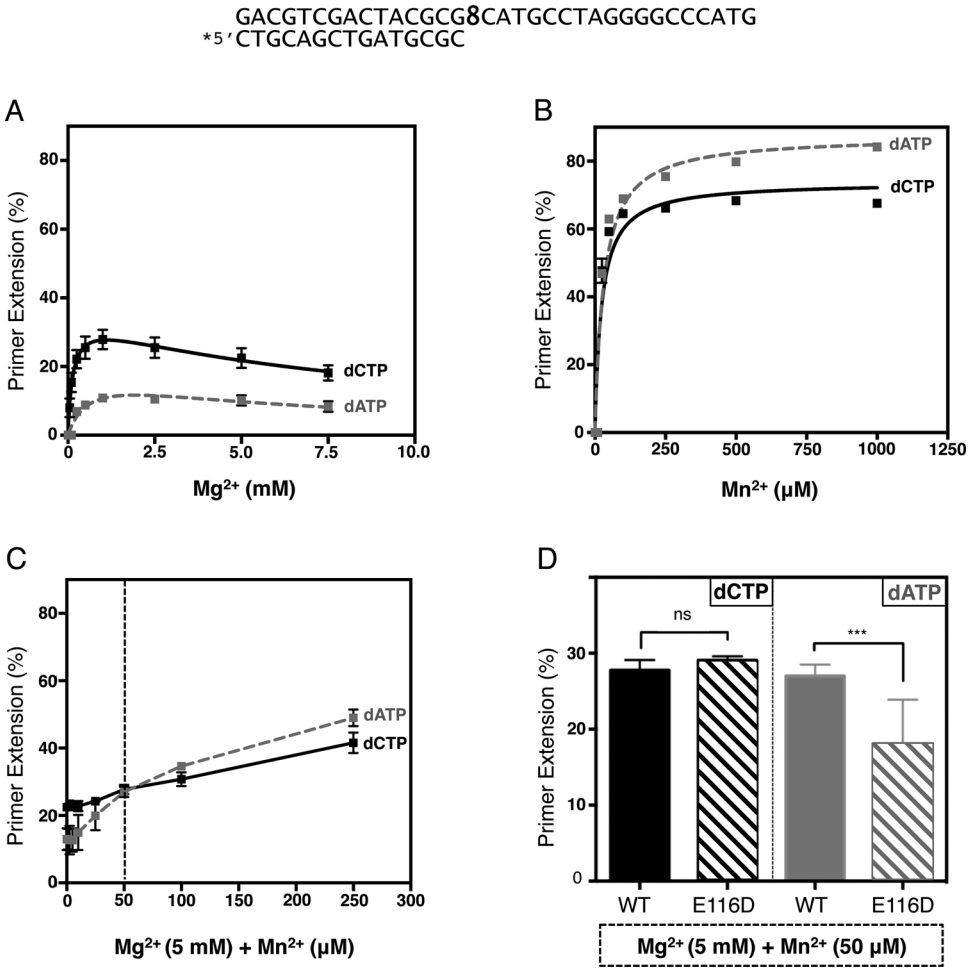


Fig. 3. Effect of different metal cofactor conditions on 8oxodG tolerance by human PrimPol WT and E116D mutant. dATP (grey dotted line) and dCTP (black line) incorporation opposite 8oxodG by purified wild-type PrimPol opposite 8oxodG in a 5′ labeled template/primer DNA structure using the indicated concentrations of: (A) Mg^{2+}, (B) Mn^{2+} and (C) 5 mM of Mg^{2+} and different Mn^{2+} concentrations. Data are represented as mean ± SD (n ≥ 2). (D) Graphic representation of dCTP (black bars) incorporation and dATP (grey bars) opposite 8oxodG using 50 μM Mn^{2+} and 5 mM Mg^{2+}. Data are represented as mean ± SD (n ≥ 2). ns: non-significant; *** P ≤ 0.001.

sequence contexts, and in the presence of 1 mM Mn^{2+} to maximize detection. Fig. 4A shows that E116D, in contrast to WT PrimPol, did not produce unscheduled expansion of the primer (Fig. 4Ab) when providing increasing concentrations of a single nucleotide (dGTP). PrimPol normally extends the primer strand by copying the first available templating base; however, when the correct nucleotide is not provided (or when the template contains a non-readable lesion), PrimPol can copy a next base by dislocating the invalid template, which is induced by the presence of the next incoming complementary nucleotide [11]. Fig. 4B shows that this type of reaction (a), and the subsequent mismatch extension (b), is about 5-fold less efficient for the E116D mutant. In a different sequence context, Fig. 4C illustrates the selectivity to incorporate the correct/complementary nucleotide versus the other three "non-complementary" nucleotides, and how the result is influenced by each metal cofactor, and the presence of Glu^{116}. When using 5 mM Mg^{2+}, only the correct incorporation (dC) is observed, at similarly low levels with both WT and E116D mutant PrimPol (Fig. 4C, left panels). When using 1 mM Mn^{2+}, the correct insertion (dC; see scheme at Fig. 4Ca) is strongly and similarly stimulated either with WT or E116D; conversely, both misinsertion of dT opposite dG (see scheme at Fig. 4Cc), and reiterative insertion of dG produced by primer realignment and expansion (see scheme at Fig. 4Cb), are now visible, but reduced in mutant E116D relative to WT PrimPol (Fig. 4C).

Unlike 8oxodG, certain DNA lesions such as AP sites, pyrimidine dimers and εA cannot be directly “read” by PrimPol. Under these circumstances, PrimPol is able to realign the primer beyond the damaged site, taking advantage of any microhomologies in the template and in the presence of Mn^{2+} ions [7,11,29]. As previously shown, this peculiar capacity of PrimPol does not require the existence of a template lesion, and can be invoked by restricting the available nucleotides [4,11]. Thus, to evaluate a distant primer realignment event mediated by PrimPol, we used a labeled synthetic dsDNA molecule whose template strand has two tetranucleotide repeats (3´CTAC5´) separated by two nucleotides (TT), and a primer strand whose 3´-terminus fully complements the first of the two repeats (see the scheme at Fig. 4D). When using Mn^{2+}, wild-type PrimPol incorporated dATP (regular primer extension, Fig. 4D left panel) much more efficiently than dCTP (via primer realignment, Fig. 4D central panel) at all metal concentrations tested. In fact, dCTP incorporation required Mn^{2+} concentrations higher than 250 μM, demonstrating that although PrimPol can promote primer realignment, it is kinetically challenging and, as expected, no dCTP insertion / primer realignment was observed at 5 mM Mg^{2+} (Fig. 4D right panel). Interestingly, the E116D variant could also promote primer realignment (dCTP insertion, Fig. 4D central panel) in the presence of Mn^{2+} ions, but with a 3-fold lower efficiency than WT PrimPol, whereas this mutation weakly affected regular primer extension (dATP incorporation, Fig. 4D left panel) in this substrate.

Despite we are using high Mn^{2+} concentrations, distant from the physiological levels [35] to maximize detection of these dislocation events "in vitro", our data demonstrate that PrimPol strictly requires Mn^{2+} to insert nucleotides onto realigned primers. Moreover, these peculiar Mn^{2+}-dependent PrimPol TLS abilities are favored by Glu^{116}, the distinctive aminoacid forming DxE motif in human PrimPol.

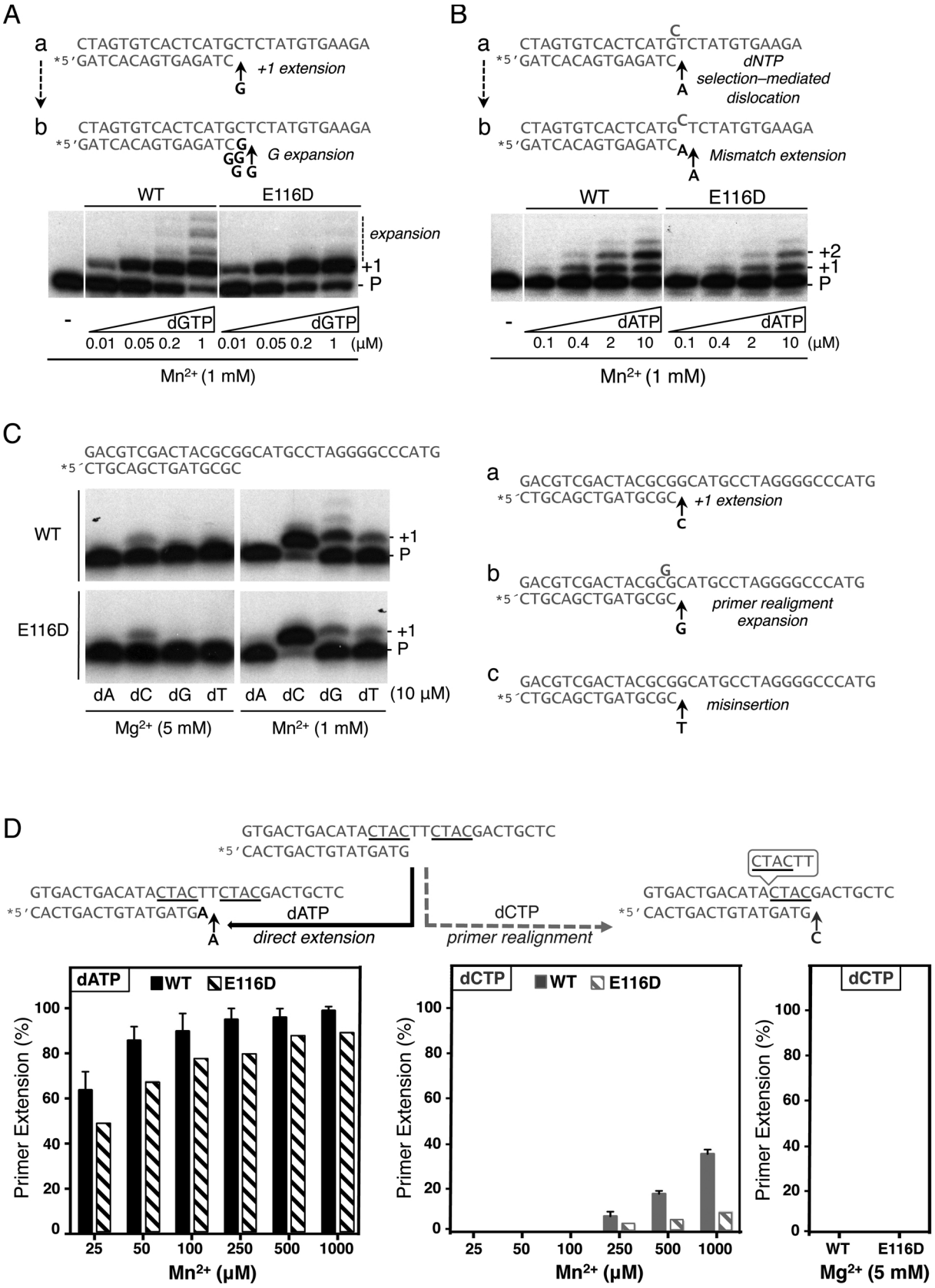
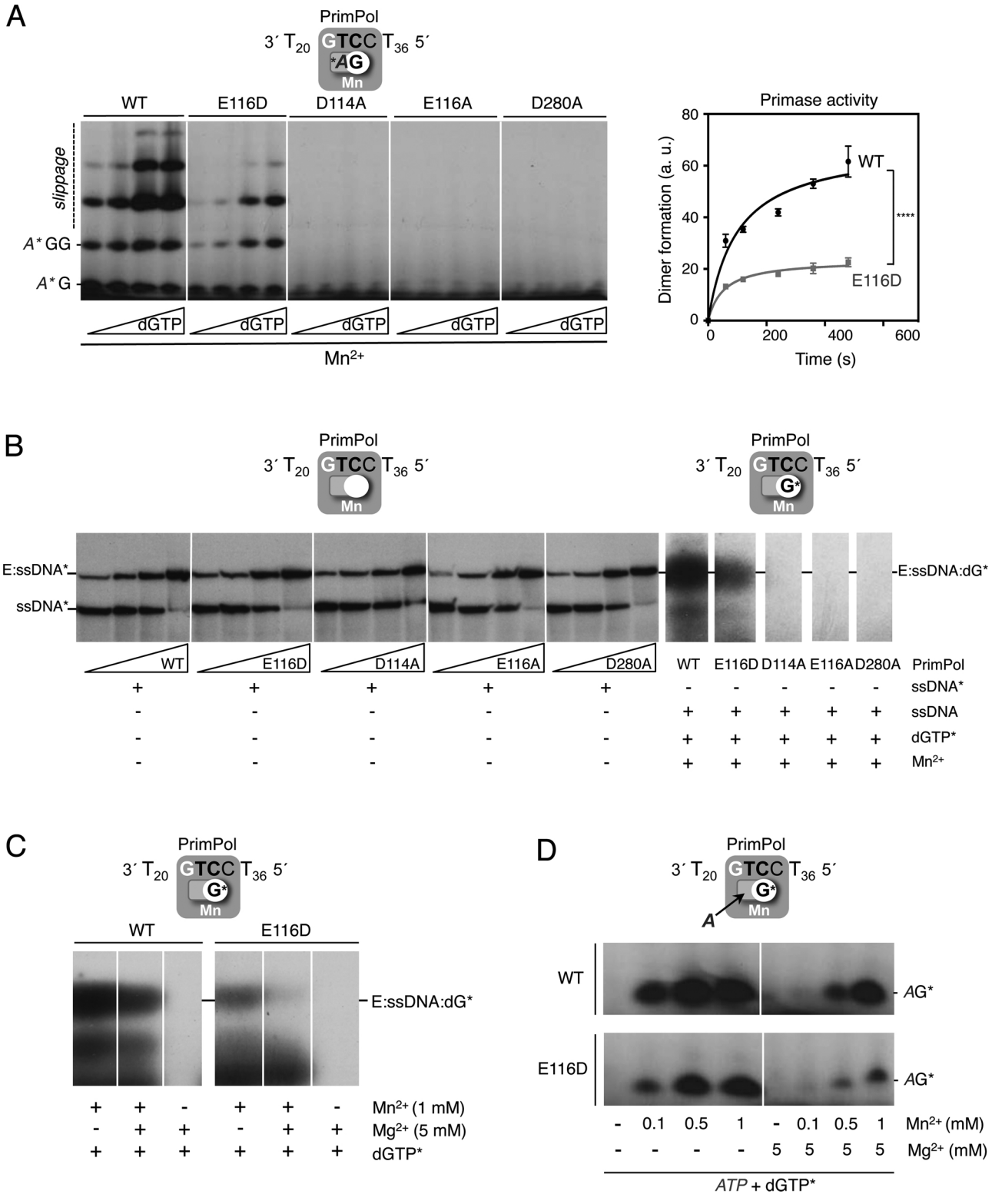


Fig. 4. Primer and template dislocation events mediated by PrimPol E116D variant. (A) Incorporation of increasing concentration (0.01, 0.05, 0.2, 1 μM) of dGTP, as the correct nucleotide opposite an undamaged template strand (2.5 nM) to produce +1 extension of the primer (a) and G expansion (b) by wild-type or E116D variant PrimPol (200 nM) in the presence of 1 mM $MnCl_2$. (B) Incorporation of increasing concentration (0.1, 0.4, 2, 10 μM) of dATP to produce template dislocation mediated by *dNTP-selection* (a), creating a dislocation error and mismatch extension of the primer (b) as a consequence by wild-type or E116D variant PrimPol (200 nM) in the presence of 1 mM $MnCl_2$. (C) Incorporation of each individual dNTP (10 μM) in front of an undamaged template (2.5 nM), providing 200 nM of either wild-type PrimPol or E116D variant in the presence of 1 mM $MnCl_2$ or 5 mM $MgCl_2$. (D) Comparatively insertion study of dATP (direct primer extension, black bars) or dCTP (primer realignment, grey bars) by wild-type PrimPol and E116D variant in the presence of either Mn^{2+} (25, 50, 100, 250, 1000 μM) or Mg^{2+} (5 mM).



(caption on next page)

Fig. 5. Characterization of metal binding ligands on PrimPol-mediated primer synthesis steps. Effect of Mn^{2+} and Mg^{2+} concentration and combination on dimer formation by PrimPol E116D variant. (A) Representative primase assay of wild-type PrimPol or mutants E116D, D114 A, E116 A, D280 A (400 nM) on GTCC template (60-mer, 1 μM) in the presence of 1 mM $MnCl_2$. The reaction contained [γ-^{32}P]ATP (16 nM) and increasing concentrations of dGTP (1, 10, 100 μM) as nucleotide substrates. Quantification of dimer formation by wild-type PrimPol or E116D variant (400 nM) on 3′GTCA5′ (1 μM) sequence, in the presence of 1 mM $MnCl_2$ providing dGTP (1 μM) and [γ-^{32}P]ATP (16 nM) at different times of reaction (0, 60, 120, 240, 360, 480 s) is shown at the right. Data are represented as mean ± SD (n = 3); **** P ≤ 0.0001. (B) Left: EMSA showing the interaction of wild-type PrimPol or mutants E116D, D114 A, E116 A, D280 A (10, 20, 40, 80 nM) with [γ-^{32}P]-labeled GTCC oligonucleotide (60-mer; 1 nM) in the absence of metal cofactor. Right: formation of pre-ternary complex when providing wild-type PrimPol or mutants E116D, D114 A, E116 A, D280 A (0.5 μM), non-labeled ssDNA GTCC (1 μM), $MnCl_2$ (1 mM), and labeled 3′-nucleotide [α-^{32}P]dGTP (16 nM). (C) Pre-ternary complex formation (E:ssDNA:dG*) when providing wild-type PrimPol or E116D variant (0.5 μM), non-labeled ssDNA GTCC (1 μM), labeled 3′-nucleotide [α-^{32}P]dGTP (16 nM) in the presence of $MnCl_2$ (1 mM); $MnCl_2$ (1 mM) + $MgCl_2$ (5 mM) or $MgCl_2$ (5 mM). (D) Dimer formation by wild-type PrimPol or E116D variant (400 nM) on 3′GTCA5′ (1 μM) sequence providing dGTP (1 μM) and [γ-^{32}P]ATP (16 nM) in the presence of $MnCl_2$ (0.1, 0.5, 1 mM); $MgCl_2$ (5 mM) or $MnCl_2$ (0.1, 0.5, 1 mM) + $MgCl_2$ (5 mM).

3.4. Human PrimPol Glu^{116} is required for efficient primer synthesis

In addition to its DNA polymerase and TLS abilities, human PrimPol is featured with the ability to synthesize DNA primers "de novo", in a reaction that is largely activated by Mn^{2+} ions [7]. Primase activity can be tested by using ssDNA template containing a preferred priming site (3′GTCC5′), labeled ATP as the 5′-site nucleotide and dGTP (1, 10, 100 μM) as the 3′-site nucleotide, in the presence of 1 mM $MnCl_2$. Under these conditions, WT PrimPol produced *3pA*dG dimers, *3pA*dGdG trimers, and longer products due to reiterative dGTP insertion via slippage (Fig. 5A). As expected, individual mutation to alanine of each putative metal ligand of human PrimPol (D114 A, E116 A, D280 A) completely ablated Mn^{2+}-dependent PrimPol primase activity (Fig. 5A). Strikingly, in clear contrast to the slightly reduced polymerase activity showed by mutant E116D (Fig. 2), this variant showed a large reduction in primase activity (Fig. 5A). When dimer formation (the initial and rate-limiting catalytic step during primer synthesis) was quantified as a function of time, the E116D variant showed 2.8-fold decrease compared to WT PrimPol (Fig. 5A, right panel).

3.5. Human PrimPol metal ligands are irrelevant for template binding

Recently we demonstrated the sequential steps and substrate preference during primer synthesis by human PrimPol and the relevance of the Zn-finger domain at each individual step [6]. Human PrimPol initially binds the ssDNA template (binary complex), then the 3'-site nucleotide to form a pre-ternary complex, followed by binding of the 5'-site nucleotide to trigger dimer formation and subsequent elongation building-up a mature DNA primer [6]. To test the involvement of human PrimPol's metal ligand residues (Asp^{114}, Glu^{116}, Asp^{280}) on these sequential steps preceding dimer formation, we first evaluated their capacity to form the binary complex (PrimPol:ssDNA). Electrophoretic Mobility Shift Assays (EMSA) involving labeled ssDNA harboring the preferred priming site 3′GTCC5′ [7] were carried out in the absence of metal ions as they were shown to be irrelevant for this step [6]. As expected, a single retarded band corresponding to the binary complex was formed at a similar protein concentration when using any metal-ligand mutants E116D, D114 A, E116 A, or D280 A (Fig. 5B left panel), implying that none of these residues (Asp^{114}, Glu^{116}, Asp^{280}) of human PrimPol are implicated in ssDNA binding.

3.6. The three metal ligands of human PrimPol are required to form a stable enzyme:ssDNA:dNTP pre-ternary complex. A change of Glu^{116} to aspartate has a negative impact on this step

After formation of enzyme:ssDNA binary complex, human PrimPol firstly binds the 3′-site nucleotide (elongation site) forming the so-called pre-ternary complex (PrimPol:ssDNA:dGTP), which can be detected exclusively in the presence of Mn^{2+} ions [6]. Moreover, it has been already demonstrated that elimination of the two carboxylates at motif A (mutant D114 A/E116 A) precludes binding of the incoming nucleotide at the 3′-site [6]. To test the importance of each single metal ligand of human PrimPol to form the pre-ternary complex, we used EMSA to assess the tripartite interaction of each individual mutant with the 3′GTCC5′-containing ssDNA template and labeled dGTP, in the presence of 1 mM $MnCl_2$. As shown in Fig. 5B (right panel), WT PrimPol formed a labeled band corresponding to the enzyme:ssDNA:dGTP pre-ternary complex, running at a similar position to that of the enzyme:ssDNA binary complex. Interestingly, the individual change of each putative metal ligand to Ala (D114 A, E116 A, D280 A) impeded formation of the pre-ternary complex (Fig. 5B right panel), explaining their null ability to catalyze the synthesis of the initial dimer (Fig. 5A). Furthermore, we evidenced that the E116D variant could form the pre-ternary complex although 2.8-fold less efficiently than WT PrimPol (Fig. 5B, right panel), in perfect agreement with its 2.8-fold lower ability to form the initial dimer (Fig. 5A, right panel).

These results emphasize the strict need of each carboxylate (Asp^{114}, Glu^{116}, Asp^{280}) for a metal-dependent stabilization of the incoming nucleotide at the 3′-site of human PrimPol, very likely implying that the two metal ions are required to attain a stable and catalytically competent configuration of the pre-ternary complex, as it has been described in *Mt*PolDom, a closely related member of the AEP superfamily [36].

3.7. Human PrimPol Glu^{116} is required for priming in the presence of two different metal ions

As shown here and in our previous work [6], a stable pre-ternary complex formed by PrimPol with ssDNA and the 3′-site nucleotide requires the presence of Mn^{2+} ions, and it could not be detected in the absence of metal or by adding Mg^{2+} ions. Nevertheless, the consequences of combining these metal cofactors, which would be the most physiological scenario for PrimPol-mediated reactions, had not yet considered. Moreover, the importance of the distinctive glutamate residue at motif A of eukaryotic PrimPols has to be evaluated also in this "two different metal ion" context during pre-ternary complex formation and subsequent dinucleotide synthesis.

Firstly, we tested the effect of the combination of both Mg^{2+} and Mn^{2+} metal cofactors on the stabilization of the 3′-nucleotide, by evaluating the formation of the pre-ternary complex as an earlier intermediate of the primase reaction. As expected, Mg^{2+} was not valid as a single metal to form the pre-ternary complex by either WT PrimPol or mutant E116D (Fig. 5C). Interestingly, the ability of 1 mM Mn^{2+} to enhance pre-ternary complex stabilization by WT PrimPol experienced a 2-fold reduction in the presence of 5 mM Mg^{2+} and 1 mM Mn^{2+} (Fig. 5C). As shown before in this study, the E116D mutant could form the pre-ternary complex in the presence of 1 mM Mn^{2+}, although 2.8-fold less efficiently than WT PrimPol (Fig. 5BC). Remarkably, the E116D mutation seems to affect even more the stabilization of the 3′-site incoming nucleotide in the presence of both metal cofactors (1 mM Mn^{2+} and 5 mM Mg^{2+}), being the pre-ternary complex (E116D:ssDNA:dGTP) almost undetectable (Fig. 5C).

Next, we evaluated the combination of both metal cofactors during dimer synthesis with ATP and labeled dGTP, templated by ssDNA containing a unique 3′GTCA5′ priming site, and using variable Mn^{2+} concentrations (0.1, 0.5, 1 mM) and a fixed Mg^{2+} concentration

(5 mM). Under these conditions, WT PrimPol primase activity (*AG* dimer formation) showed a general inhibitory effect by Mg^{2+} ions at all Mn^{2+} concentrations tested (Fig. 5D). As expected, neither WT nor E116D PrimPol was unable to form the dimer in the presence of Mg^{2+} (Fig. 5D). Notably, Glu^{116} mutation to aspartate reduced dimer synthesis in all conditions tested, more markedly when both metal cofactors were present (Fig. 5D).

Collectively, our data validate the strict requirement of Mn^{2+} ions to promote a stable binding of the 3′-site incoming nucleotide, required during primer synthesis by human PrimPol. These results also support the critical role of the distinctive carboxylate residue of the DxE motif (Glu^{116} in human PrimPol), most likely favoring the use of Mn^{2+} as the metal cofactor.

4. Discussion

A crucial aspect of the "two-metal-ion catalytic mechanism" of polymerization shared by polymerases and primases is the precise interaction of two (identical or different?) metal ions with the three conserved catalytic carboxylates to allow the proper alignment of the attacking nucleophile and the alpha-phosphorus of the incoming nucleotide. Metal ion A promotes formation of the attacking nucleophile, and metal ion B binds to and facilitates the leaving of the β and γ phosphates [16,22]. The present study further emphasizes the importance of these metals and their ligands for overall catalysis, and their mutual collaboration to stabilize the incoming nucleotide at the 3′-site of human PrimPol. We showed here that substitution of each carboxylic residue (Asp^{114}, Glu^{116}, Asp^{280}) with alanine does not affect PrimPol's binding to ssDNA, but abolishes its the stabilization of the 3´-nucleotide by forming the PrimPol:ssDNA binary complex, a pre-catalytic step required to initiate primer synthesis.

Thus, it is tempting to speculate that a precise network of interactions between the three carboxylates acting as metal ligands and the two metal ions (at sites A, B) is essential for the stabilization of the incoming nucleotide at the 3′-site of human PrimPol, as a crucial intermediate preceding dimer formation. Importantly, one of these metal-ligand carboxylates is invariantly a glutamate in eukaryotic PrimPols (motif DxE), unlike the more conventional aspartate found in other primases and polymerases. A mutation of Glu^{116} to Asp in human PrimPol strongly affected the formation of the pre-ternary complex, especially in the presence of both Mg^{2+} and Mn^{2+}.

Recently, the crystal structure of the catalytic core of human PrimPol has been resolved in complex with a template/primer, one metal ion (Ca^{2+}) which occupies the metal B position, and dATP as incoming 3′-site nucleotide but, significantly lacking the second metal ion (A) at the active site [31]; see Fig. 6AB). Ca^{2+} does not support PrimPol's catalysis [12], most likely not reflecting the proper coordination of metal ions at PrimPol's active site. In spite of these caveats, the crystal structure shows that the invariant Asp^{280} of motif C does not make specific contacts with the incoming nucleotide (Fig. 6 C), suggesting its catalytic role as a direct ligand of metal A (absent in this crystal), and its indirect contribution to the interacting network that stabilizes the 3´-nucleotide. Conversely, the invariant Asp^{114} of DxE motif A shows interactions with the 3′-site incoming nucleotide through its α and β phosphate groups (Fig. 6C), which would explain the adverse effects observed in this study for the D114 A catalytically-dead mutant. On the other hand, the critical role of the invariant Glu^{116} of DxE motif, evaluated here in detail, is also supported by the direct interactions shown at the crystal structure. As shown in Fig. 6C, the distinctive residue Glu^{116} interacts with metal B and with the α and γ phosphate groups of the 3′-site incoming nucleotide, but also can establish interactions with two highly conserved PrimPol residues, Lys^{165}

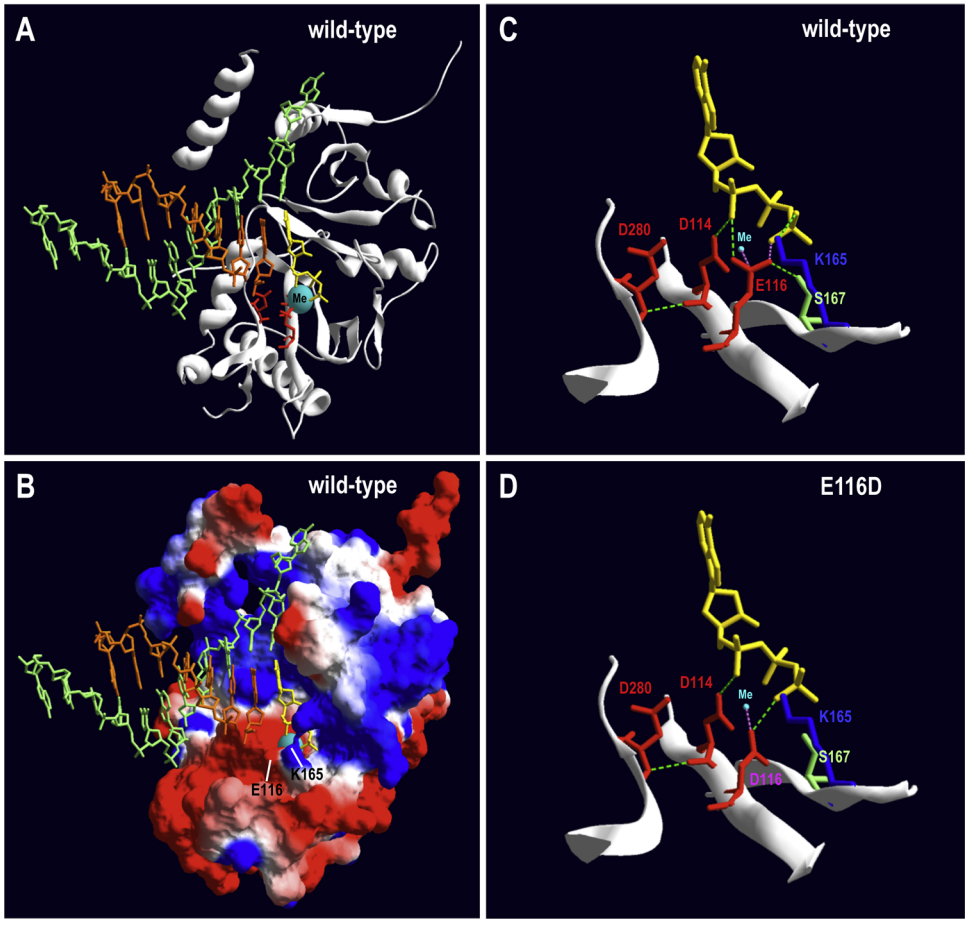


Fig. 6. Crystal Structure details of human PrimPol in complex with template/primer, dATP and Ca^{2+}. (A) Ribbon representation of the quaternary complex of *Hs*PrimPol:DNA:dATP depicting the interaction of PrimPol (white) with the dsDNA and dATP (yellow) by the catalytic residues Asp^{114}, Glu^{116}, Asp^{280} (red). DNA is colored orange for primer strand and green for templating strand. The metal ion is cyan (Me). (B) Electrostatic surface potential in the active site pocket of *Hs*PrimPol. (C) A close-up view of the active site of *Hs*PrimPol highlighting the catalytic residues and their specific interactions with the 3′-site incoming nucleotide and the metal cofactor colored as in (A). (D) Extrapolation of E116D variant (pink) and its different interactions. 3D images and modeling of mutated residues were created with Swiss-PdbViewer (DeepView) program, using *Hs*PrimPol PDB ID: 5L2X, which corresponds to the crystal structure of human PrimPol ternary complex [31].

and Ser^{167}, which are also ligands of the γ phosphate of the 3′-site incoming nucleotide (see also Fig. 6B). Modeling of the E116D mutation (Fig. 6D) outlines the potential loss of some of these contacts (α phosphate, Lys^{165} and Ser^{167}), in spite of the maintained interaction with metal B and γ phosphate of the 3′-site incoming nucleotide. Hence, our results corroborate the structural data concerning the critical role of the three catalytic carboxylates and emphasize the importance of Glu^{116} in promoting the use of Mn^{2+} to stabilize the pre-ternary complex.

Carboxylates coordinating metal ion B have been shown to be more sensitive to mutations than those coordinating metal ion A, as the B site is suggested to exhibit stronger metal ion preference than the A site [16,37,38]. Thus, in the absence of more precise structural information, we speculate that Glu^{116} residue (motif DxE) of human PrimPol may coordinate Mn^{2+} as metal ion B. Interestingly, isothermal titration calorimetry experiments (ITC) revealed that Mn^{2+} (but not Mg^{2+}) significantly stimulates binding of nucleotides to human primase, in agreement with structural data of the ternary complex (p48AL:UTP:Mn^{2+}) showing multiple stabilizing interactions between the metal ion, the incoming nucleotide and the enzyme [39]. It is commonly accepted that Mn^{2+} ions, which have more options of coordination than Mg^{2+}, is the optimal ion to stabilize unconventional intrahelical conformations of the incoming dNTPs, as those occurring when dNTPs are inserted opposite damaged templates [23]. The structural basis for the enhanced catalytic activity of PrimPol in the presence of Mn^{2+} is presently unknown, but our biochemical data allow us to speculate that Mn^{2+} may allow greater flexibility of PrimPol active site to adopt an optimal conformation to stabilize the 3´-incoming nucleotide during TLS events mediated by primer/template realignments, especially during formation of the primase pre-ternary complex.

The biological relevance of Mn^{2+} as the metal cofactor for the in vivo function of primases and DNA polymerases remains elusive. In a recent paper [40], Zhao and coworkers used enzyme kinetic analyses and computer simulations to dissect the mechanism by which PrimPol transfers a nucleotide to a primer-template DNA, concluding that divalent cations alter the rate-limiting step of PrimPol-catalyzed DNA elongation. According to the authors, in the presence of Mn^{2+}, a conformational transition step from non-productive to productive PrimPol:DNA complexes limits the enzymatic turnover, whereas, in the presence of Mg^{2+}, the chemical step becomes rate limiting. Both kinetic data and simulations support the notion that Mn^{2+} can be a preferred metal cofactor for PrimPol, even under micromolar Mn^{2+} concentration [41]. As stressed by the authors, their conclusion can be extrapolated to the elongation stage of PrimPol-catalyzed priming, but further studies will be required to address the kinetic steps during dinucleotide synthesis and their dependence on Mn^{2+} ions.

The concentration of free Mn^{2+} is low and is tightly regulated in vivo by special metal ion binding proteins [41]; however, some studies point out the possibility that there is an intracellular increase of Mn^{2+} under oxidative stress conditions [42]. This increase is required to trigger a superoxide dismutase-dependent (using Mn^{2+} as a cofactor) antioxidant strategy, and to stimulate certain enzymes (such as PrimPol), whose active centers could have evolved to use Mn^{2+} to facilitate rate limiting synthetic reactions, where efficiency is more important than fidelity.

5. Conclusion

In this work we have explored the metal ligand residues at human PrimPol active site, to understand the preferential use of manganese to boost the most distinctive PrimPol reactions: DNA priming and TLS abilities. Our results explain why the conserved motif A (DxD in most primases belonging to the AEP superfamily) is modified in PrimPols to be DxE. That glutamate residue (Glu^{116}) contributes to error-prone tolerance of 8oxodG and is also relevant in TLS events mediated by primer/template realignments. Moreover, we showed that Glu^{116} is crucial for using Mn^{2+} ions to achieve optimal incoming nucleotide stabilization, a primary step required during initiation of primer synthesis.

Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2019.03.006.

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