DNA polymerase (pol) μ is a DNA-dependent polymerase that incorporates nucleotides during gap-filling synthesis in the non-homologous end-joining pathway of double-strand break repair. Here we report time-lapse X-ray crystallography snapshots of catalytic events during gap-filling DNA synthesis by pol μ. Unique catalytic intermediates and active site conformational changes that underlie catalysis are uncovered, and a transient third (product) metal ion is observed in the product state. The product manganese coordinates phosphate oxygens of the inserted nucleotide and PPi. The product metal is not observed during DNA synthesis in the presence of magnesium. Kinetic analyses indicate that manganese increases the rate constant for deoxynucleoside 5′-triphosphate insertion compared to magnesium. The likely product stabilization role of the manganese product metal in pol μ is discussed. These observations provide insight on structural attributes of this X-family double-strand break repair polymerase that impact its biological function in genome maintenance.
DNA synthesis is a fundamental reaction required for essential processes such as DNA replication and repair. DNA polymerases catalyze incorporation of a deoxynucleoside 5′-triphosphate (dNTP) at the end of a primer DNA strand, extending the primer by one nucleotide (dNMP) and producing pyrophosphate (PP). Historically, DNA polymerases have been thought to utilize a two-metal mechanism that is shared by other nucleotidyl transferase enzymes.

However, many important features of the catalytic mechanism of DNA polymerases have remained elusive, in part, due to a lack of high-resolution crystal structures of intermediate states during the catalytic cycle. Recent structural descriptions of nucleotide insertion by DNA polymerases and terminal deoxynucleotidyl transferase (TdT) employed time-lapse X-ray crystallography to capture snapshots of catalysis within the crystal and reveal novel intermediates during the catalytic cycle. This approach employs natural substrates, instead of substrate analogs, to follow the reaction in crystallo and provides structures of the liganded enzyme before, during, and after catalysis. Thus, time-lapse X-ray crystallography can reveal conformational adjustments and structural features as a polymerase (pol) transitions from one liganded form to another. In particular, an adjacent third (product) metal was observed to coordinate product phosphate atoms during time-lapse analysis of pol β along with loss of the essential active site catalytic metal.

Although additional metals of unknown function were previously observed near the active sites of DNA and RNA polymerases, computational studies with pol β suggested that the third (product) metal does not lower the energy barrier for the forward reaction but suppresses the reverse reaction. In the case of pol η, it has been suggested that the third (product) metal promotes the forward reaction and may facilitate product release.

Based on sequence homology, the mammalian DNA polymerases are classified into families: A, B, X, Y, and reverse transcriptase. The X-family members share a common carboxyl-terminal pol domain with significant sequence and structural homology. Single-nucleotide gap filling by pol β, the smallest eukaryotic X-family member, has been extensively characterized both structurally and kinetically. In brief, an “open” pol/DNA binary complex binds the incoming dNTP along with a magnesium ion, referred to as the “nucleotide” metal (Mg). This binding event triggers formation of the “closed” ternary complex. The “closing” transition repositions the carboxyl-terminal N-subdomain to sandwich the nascent base pair between polymerase side chains and the duplex DNA terminus.

Binding of the catalytic magnesium (Mg) results in formation of the pre-catalytic “ground state” ternary complex, with two metals, poised for chemistry. The catalytic metal ion facilitates deprotonation of the deoxyribose 3′-hydroxyl group of the primer strand. Nucleotidyl transfer through an in-line attack of the α-phosphate of the incoming dNTP results in phosphodiester bond formation and breaking of the Pα–Oα bond of the incoming nucleotide, generating PP. After chemistry, conformational changes occur resulting in polymerase “opening” and release of products.

Pol μ functions in non-homologous end joining (NHEJ) DNA repair, a major double-strand break repair pathway. Among X-family members, pol μ is most closely related to TdT (41% identity between human paralogs) and possesses the ability to bridge non-complementary DNA ends during NHEJ.

Fig. 1 Global conformational changes ($\Delta C_v$) upon binary-to-ternary complex transition in (a) pol μ and (b) pol β. The structures shown are the ternary complexes of each enzyme. The protein backbones are colored as a heat map from blue to red corresponding to changes in $C_v$ positions on a scale from 0 to 1 Å, calculated from an alignment of the binary (PDBe IDs 4LZG and 3ISM for pol μ and pol β, respectively) and ternary (PDBe IDs 4M04 and 2FMS for pol μ and pol β, respectively) complexes using the Matchmaker tool in the Chimera package. All the $C_v$ of the respective pol μ structures were used to perform the alignment, as pol μ does not display subdomain motions (RMSD, 0.23 Å).

In contrast, since the N-subdomain of pol β undergoes repositioning when a nucleotide binds to form the ternary complex (Fig. 4a in ref. 20), the alignment was restricted to the other pol β subdomains (RMSD, 0.86 Å, 224 residues). The domains/subdomains are indicated: the polymerase domain includes the D+, C+, and N-subdomains corresponding to the thumb, palm, and fingers of right-handed polymerases, as well as the 8-kDa and lyase (L) domain of pol μ and β, respectively. Active site metals are shown as green spheres and the ends of the disordered loop region in pol μ are shown connected by a dashed line (cyan).

Results

Time-lapse X-ray crystallography. We applied time-lapse crystallography to visualize single-nucleotide insertion by pol μ in the presence of either Mg$^{2+}$ or Mn$^{2+}$ using a single-nucleotide gapped DNA substrate; this substrate had been used previously to structurally characterize pre- and post-catalytic complexes.

In addition, a similar gapped DNA was used in time-lapse crystallography with pol β, a model X-family polymerase. Initially, a pre-catalytic ground state (GS) ternary substrate complex was formed in crystallo (Fig. 2a). To accomplish this, a crystal of the pol μ/DNA binary complex was obtained in the absence of a divalent metal ion, and then the crystal was soaked in a cryo-solution containing CaCl$_2$ and dNTP for 15 min at 4°C; note that calcium does not support catalysis. The Ca$^{2+}$-bound GS crystal was transferred to a cryo-solution containing Mg$^{2+}$ or Mn$^{2+}$. The bound Ca$^{2+}$ is replaced by Mg$^{2+}$ or Mn$^{2+}$, which support catalysis, thereby initiating the
The pre-catalytic GS ternary complex. A 1.95 Å structure of the pre-catalytic GS ternary complex of pol μ was obtained after the soak in Ca²⁺ and TTP (Supplementary Table 1). The active site is fully occupied with the incoming TTP (opposite templating base adenine) and Ca²⁺ atoms are observed in the catalytic (Ca) and nucleotide metal (Ca₄) binding sites (Fig. 2b). No incorporation of the incoming nucleotide was evident in the GS structure, as revealed by the lack of electron density between O₃α and P₀ of the incoming nucleotide. Apart from minor coordination distance differences due to the identity of the metal (Ca²⁺ vs. Mg²⁺), the GS structure is very similar to the reported structure of pol μ with distances (Å) indicated. The distance between O₃α and P₀ is shown as a red dashed line.

Mg²⁺-mediated insertion. In order to observe structural changes that accompany the insertion of dNMP into gapped DNA by pol μ, the Ca²⁺ ions occupying the catalytic and nucleotide metal sites were exchanged for a divalent metal that supports catalysis. We soaked crystals of the GS pre-catalytic ternary complex in a cryo-solution containing 10 mM MgCl₂ at 4 °C, initiating catalysis in crystallo. After a 15 min soak, the crystals were flash frozen and the structure determined to 2.10 Å (Supplementary Table 1). The structure at this stage of the reaction was very similar to the structure of the GS ternary complex. However, as shown in the F₀−Fₛ simulated annealing omit map (Fig. 3a), density is observed between O₃α and P₀ (TTP) and between Pₜ and Pβ indicating that at this time point some product formation had occurred and that some reactant persisted. Clear inversion at P₀ can be observed, indicative of bond formation; occupancy refinement of both the incorporated and incoming nucleotides indicates ~50–60% incorporation of TTP occurred. This is defined as the reaction state (RS). The product PPᵢ, with an occupancy of ~50–60%, is located in a position identical to that expected directly after bond cleavage. Subtle changes in active site side-chain conformations are observed, including the observation that a proportion of His329 has flipped ~150° into a distal conformation and now lies ~5 Å away from Py (Fig. 4a). The occupancy of the distal conformation of His329 corresponds to
the amount of PP$_i$ formed and dNMP incorporated (~50–60%). Bond lengths, angles, and coordination geometry are consistent with occupancy of the catalytic and nucleotide metal-binding sites with Mg$^{2+}$, along with some Ca$^{2+}$ (~50%) in the catalytic metal-binding site due to incomplete exchange with Mg$^{2+}$. Although a third or product metal was seen to coordinate the product phosphates, DNA +1 and PP$_i$, in the product state. The position of Pβ of the PP$_i$ product is identical to that expected after bond cleavage, but the oxygen ligand of Py is more disordered, with a water replacing the interaction with Mg$_n$. Thus, density remains for Pβ, suggesting that Py dissociates prior to Pβ to facilitate product release (Fig. 3c). His329 flips ~150° into the distal conformation and Asp330 rotates ~90° to adopt a post-catalytic conformation (Fig. 4a). Based on coordination distances and geometry, the nucleotide metal-binding site is fully occupied by Mg$^{2+}$ coordinating the PP, leaving group oxygens, Asp330 and Asp332, as well as a water molecule. The atom in the catalytic metal-binding site, however, now possesses longer coordination distances (~2.1 Å) with loss of the O3′ coordinating ligand, consistent with Na$^+$ at this site. Sodium has therefore partially replaced Mg$^{2+}$ in the catalytic site, in a fashion similar to that at the corresponding site during the pol β catalytic cycle.

To observe post-catalytic events in the pol μ reaction cycle (e.g., PP, and metal release), we performed extended soaks, up to 960 min, of the product complex. The structures were generally similar to the product structure after the 60 min soak. A 1.97 Å structure was obtained after a 270 min soak and displayed full turnover (Supplementary Fig. 2a, b). The nucleotide metal site is occupied by Mg$^{2+}$, and the catalytic site is now fully occupied with Na$^+$. A 960 min soak structure (PS*) was determined at 1.73 Å, and the structure is shown in Supplementary Fig. 3. Based on coordination distances and geometry, the nucleotide metal-binding site is occupied by Mg$^{2+}$, while Na$^+$ still occupies the catalytic metal site. His329 is in the distal conformation, while

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**Fig. 3** Magnesium-mediated insertion. Soaking of the Ca$^{2+}$-bound pre-catalytic GS ternary complex in a cryo-solution containing 10 mM MgCl$_2$ initiates the reaction in the crystal. Crystals were frozen after incubation for the indicated times in the cryo-solution. a, b Reaction state (RS) at 15 min, c, d product state (PS) at 60 min. The panels on the left (a, c) display density for the incoming and incorporated nucleotides. The panels on the right (b, d) are close-up views of a 90° rotation of the active site with coordinating ligands indicated by dashed lines and labeled distances (Å). The incoming TTP and incorporated nucleotides (TMP) are in cyan and green stick representation, respectively, while the protein side chains are in yellow. Mg$^{2+}$ ions are displayed as green spheres and water molecules as blue spheres. Sodium is shown as a large purple sphere. The Fo–Fc simulated annealing omit map (green mesh) shown is contoured at 2.5σ.
Asp330 is in the post-catalytic state in both the 270 and 960 min soaks (Fig. 4a). Importantly, both extended soak product structures suggest PP\textsubscript{i} release. The density present at the PP\textsubscript{i}-binding site is consistent with a potential cryo-solution contaminant, glycolate, that was modeled into the active site. However, the presence of a small amount of PP\textsubscript{i} bound to the active site cannot be excluded.

**Mn\textsuperscript{2+}-mediated insertion.** Based on enzymatic assays, manganese has been suggested to be the physiological metal co-factor of pols \(\lambda\)-polynucleotidase. The anomalous signal produced by Mn\textsuperscript{2+} was employed to identify active site metals. Accordingly, structures determined along the reaction pathway of Mn\textsuperscript{2+}-mediated catalysis by pol \(\mu\) were obtained. A structure of pol \(\mu\) at 7.5 min after reaction initiation was solved at 1.93 Å (Supplementary Table 1). The structure at this stage of catalysis displays ~60% turnover (Fig. 5a) and is very similar to the structure of the Mg\textsuperscript{2+}-catalyzed reaction, where density for a mixture of reactant and product species was observed. In this case, however, a third (product) Mn\textsuperscript{2+} is observed coordinating P\textsubscript{α} of the incorporated nucleotide and P\textsubscript{β} of the PP\textsubscript{i} product. Four water molecules complete the octahedral coordinating metal sphere. This site possesses density attributed to Mn\textsuperscript{2+}-mediated anomalous dispersion (Fig. 6a), and its occupancy and those of the surrounding water molecules correspond to the percentage of product formation. As in the Mg\textsuperscript{2+}-mediated reaction, His329 is seen to flip into a distal conformation ~5 Å away from P\textsubscript{γ}, while its occupancy (~60%) corresponds to the amount of PP\textsubscript{i} formed. The conformation of His329 in the manganese structure is intermediate between the proximal and distal conformations, where N\textsubscript{ε}2 is within hydrogen-bonding distance of P\textsubscript{γ} (Fig. 4b). Increased dynamics in the PP\textsubscript{i} is observed, as positive density near P\textsubscript{γ} and the product metal site remained after occupancy refinement of the product PP\textsubscript{i} and product metal regions of this structure. The positive density is weak and was not modeled. The catalytic and nucleotide metal-binding sites contain octahedrally coordinated Mn\textsuperscript{2+} with characteristic coordination distances \(2.7 \pm 0.1\) Å. Structures obtained at earlier (4 min) and later (10 min) time points corresponded to ~40 and ~70% turnover, respectively (Supplementary Table 1). These structures were identical to the 7.5-min reaction state structure, except for increasing occupancies of the incorporated nucleotide, product PP\textsubscript{i}, the product metal (Mn\textsubscript{p}), and the corresponding distal conformation of His329, as the reaction progressed. Changes in the conformation of Asp330 were not observed in these structures and this residue stays in the pre-catalytic conformation throughout the Mn\textsuperscript{2+}-mediated catalytic cycle (Fig. 4b).

Figure 5c, d illustrates the active site of the product state in the Mn\textsuperscript{2+}-mediated reaction 15 min after initiating the reaction. Occupancy refinement indicates 100% occupancy of incorporated TMP with no density for a bond between P\textsubscript{α} and P\textsubscript{β} in the F\textsubscript{o}–F\textsubscript{c} simulated annealing omit map (Fig. 5c). His329 displays a distal conformation, where N\textsubscript{ε}2 is seen to coordinate P\textsubscript{γ}. Increased dynamics in PP\textsubscript{i} is again evident due to increased positive density near P\textsubscript{γ}. Asp330 has not rotated, consistent with the catalytic and nucleotide metal-binding sites being fully occupied by Mn\textsuperscript{2+}. The product metal is observed with an occupancy of ~60–70%, coordinating the phosphate of the incorporated nucleotide, P\textsubscript{β} of PP\textsubscript{i}, as well as four water molecules, as observed in the reaction state described above for the Mn\textsuperscript{2+}-partial catalysis complex.

In order to observe any post-catalytic changes associated with the Mn\textsuperscript{2+}-mediated reaction, we performed longer soaks (60 and 960 min). A 1.95 Å structure was determined following a 60 min soak (Supplementary Table 1) and is consistent with a product structure with full incorporation of TTP as shown in the F\textsubscript{o}–F\textsubscript{c} simulated annealing omit map (Supplementary Fig. 2c, d). The third (product) metal is still observed in this structure, but at a reduced (40%) occupancy, indicating the product metal has partially dissociated from the active site with the extended soak. The occupancy of PP\textsubscript{i} is 100% in this structure. The structure after a 960 min soak (PS*) also is consistent with a product structure. Surprisingly, both nucleotide and catalytic metal-binding sites still possess an anomalous signal (Fig. 6c), and the coordination distances/geometry are consistent with Mn\textsuperscript{2+}, in contrast to observations during Mg\textsuperscript{2+}-mediated catalysis, where the catalytic metal is seen to dissociate upon product formation. The arrangement of coordinating atoms is consistent with penta-coordinated stabilization of the Mn\textsuperscript{2+} ion in the catalytic metal site. The extended soak is also consistent with PP\textsubscript{i} release, where the remaining density can again be modeled as a glycolate (Supplementary Fig. 4). The product metal site is devoid of density at this stage, indicating that the third (product) metal has fully dissociated. Using the anomalous signal conferred by Mn\textsuperscript{2+} as an indicator, we observed the product metal in structures beginning from 40% product formation until long after the reaction is complete.

**Pol \(\mu\) single turnover kinetics.** Catalysis in crystallo increases with time, as expected for an enzymatic reaction, but is considerably slower than the steady-state reaction in solution at
physiological temperatures. The decreased activity in crystallo is most likely due to the lower temperature (4 °C) employed during the soaks, presence of inhibitory Ca^{2+} ions, pre-requisite ion-exchange events in a viscous cryo-solution, and other impediments that might be imposed by the crystalline nature of the reaction mixture.

To determine the pol μ nucleotidyl transferase rate, a single-turnover kinetic analysis (enzyme-DNA) of pol μ was performed with Mg^{2+} or Mn^{2+} as the metal ion co-factor. The single-turnover rate constant with single-nucleotide gapped DNA was significantly greater than that reported with a non-gapped substrate. The observed rate constants for the exponential time courses (Fig. 7a) increased hyperbolically with TTP concentration (Fig. 7b), providing estimates of the turnover rate constant with single-nucleotide gapped DNA was much greater (~190 μM) than the physiological nucleotide concentration. Substituting manganese for magnesium only modestly increased the apparent binding affinity for the correct nucleotide, as the rate of insertion was similar to that expected for a DNA repair polymerase, the apparent binding affinity for the correct nucleotide was much greater (~190 μM) than the physiological nucleotide concentration. Substituting manganese for magnesium only modestly increased the apparent binding affinity. However, k_{pol} for nucleotide insertion increased appreciably, such that overall insertion efficiency increased 50-fold relative to magnesium (Table 1).

Nucleotide analogs with sulfur replacing a non-bridging oxygen on Pα of the incoming nucleotide have been used to analyze whether a chemical step is rate limiting during single-turnover kinetic analysis. Since sulfur is less electronegative than oxygen, a decreased rate of insertion is expected with sulfur substitution if chemistry is the rate-limiting step. The measured elemental effect for magnesium-dependent nucleotide insertion (Table 1) indicates a strong thio-effect (24-fold). Interestingly, substitution with manganese suppressed the thio-effect, suggesting a change in rate-limiting step from chemistry in the magnesium-dependent insertion, to a non-chemical step in the manganese-dependent insertion.

Discussion

In this study, we applied time-lapse crystallography to characterize nucleotide insertion by pol μ. Previous time-lapse crystallographic studies of pols β and η uncovered a new active site metal (i.e., third or product metal) thought to have a catalytic role due to its coordination with products. Here we identified the presence of a product metal in the insertion reaction of pol μ. In the presence of Mn^{2+}, the product metal site was observed from an early stage (~40%) of the reaction until its completion and beyond. The occupancy of the product metal site correlated with the degree of nucleotide incorporation. After full turnover, the product metal dissociated from the active site, leaving PP_i behind. The PP_i then dissociated from the active site.

The active site of pol μ is similar to the other X-family members, especially that of pol β (Fig. 8). Six conserved active site residues in pols μ and β, including the metal-coordinating

![Fig. 5 Manganese-mediated insertion. Soaking of the Ca^{2+}-bound pre-catalytic GS ternary complex in a cryo-solution containing 10 mM MnCl_2 initiates the reaction in the crystal. Crystals were frozen after incubation for the indicated times in the cryo-solution. a, b Reaction state (RS) at 7.5 min, c, d product state (PS) at 15 min. The panels on the left (a, c) display density for the bond, indicating the degree of completion of the reaction. The panels on the right (b, d) are close-up views of a 90° rotation of the active site with coordinating ligands indicated by dashes and distances (Å) labeled. The incoming and incorporated nucleotides are in cyan and green stick representation, respectively, while the protein side chains are in yellow. Mn^{2+} ions are displayed as purple spheres and water molecules as blue spheres.](image-url)
contrast to observations with pol β, an adjunct third (product) Mg²⁺ is not observed to coordinate products (i.e., phosphate oxygens on the inserted nucleotide and PPi). However, similar to pol β, Mn²⁺ is observed to coordinate product oxygens. These observations suggest that a third metal is not essential for DNA synthesis.  

With Mg²⁺-dependent insertion, His329 flips ~150° into a distal conformation, the catalytic Mg²⁺ dissociates and is replaced by Na⁺ (Fig. 4a). Additionally, Asp330 rotates ~90°. In the presence of Mn²⁺, the flip of His329 is observed, but Asp330 remains in the pre-catalytic conformation and maintains its coordination with the catalytic Mn²⁺ throughout the reaction (Fig. 4b). After the Mg²⁺-dependent reaction is complete, PPi dissociates and is replaced by water molecules (Fig. 4a, right panel). Surprisingly, and unlike previous observations, the nucleotide Mg²⁺ remains bound to the enzyme after departure of PPi, indicating that PPi and nucleotide Mg²⁺ release are uncoupled.

Ligand binding to pol μ is not accompanied by large conformational changes in protein or substrates (Fig. 1a and Supplementary Fig. 5). However, as noted above, differences in side-chain positions do occur in response to metal binding and dissociation. The three active site aspartate residues, required for metal binding and catalysis, do not undergo significant changes in pol β, whereas in pol μ, Asp330 rotates 90° when the catalytic magnesium is released near the end of the reaction. Prior to the rotation of Asp330, His329 shifts from stabilizing Pγ of the incoming nucleotide to the distal conformation, flipping ~150° and repositioning Ne2 ~5 Å away from its initial position (Fig. 4a). This appears to facilitate product release. Asp330 blocks the rotation of His329 into the proximal conformation, and the flip of His329 allows rotation of Asp330 during the Mg²⁺-catalyzed reaction. This rotation of Asp330 is not observed in the presence of Mn²⁺, as the catalytic metal site remains occupied by manganese (Fig. 4b, right panel).

Manganese is suggested to be the in vivo metal co-factor of pol μ, as this enzyme is activated in vitro at physiological Mn²⁺ concentrations (i.e., 100 μM) and inhibited at physiological Mg²⁺ concentrations (1–2 mM). We observe metal rearrangements and associated metal-dependent structural transitions in the pol μ active site during catalysis. The dynamics of the catalytic and nucleotide metals in pol μ follow a similar pattern to those in pol β. Differences in metal function must exist, however, in light of pol μ’s increased Mn²⁺-dependent rate constant of insertion, relative to that with Mg²⁺. A similar rate increase for Mn²⁺ over Mg²⁺ is not seen with pol β.

Both catalytic and nucleotide metal sites are occupied from the GS to the initial PS with both Mg²⁺- and Mn²⁺-mediated reactions in crystallo. Yet, the exchange of Mg²⁺ with Na⁺ in the catalytic metal site in the Mg²⁺-catalyzed reaction follows the loss of coordination with O3’, due to bond formation, and Asp330. In contrast, the catalytic metal site remains occupied by Mn²⁺ to the end of the reaction. The stability of Mn₃ appears to preclude rotation of Asp330. The nucleotide metal site binds the nucleotide metal as soon as the nucleotide enters the active site, and stability of the nucleotide metal-binding site is indicated by low B-factors in all ternary complex structures, as well as the persistent and strong Mn²⁺ anomalous signal.

In time-lapse crystallography with pol μ in the presence of Mn²⁺, the Mn₃ was observed in a similar location as the product metal of pol β (Fig. 8). In pol β, the product metal is surrounded by four coordinating waters, while in pol μ three coordinating waters surround the Mn²⁺ (~2.2–2.4 Å) with a 4th water ~2.7–3.1 Å away (Fig. 5b,d). The product metal site in pol μ is thus less likely to bind Mg²⁺ due to its strict octahedral coordination requirement. In pol μ, the rate of nucleotide

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**Fig. 6** Anomalous density map overlayed on structures of the (a) RS, (b) PS, and (c) PS* generated by Mn²⁺-mediated anomalous dispersion. PS* refers to an extended soak (960 min) showing the product metal and PPi, have dissociated, but the catalytic and nucleotide metals remain bound. Anomalous density is shown as a pink mesh at 5σ, while the Mn²⁺ atoms are displayed as magenta spheres. The incoming nucleotide is shown in green stick representation, while DNA is shown in cyan. Distances (Å) between metals are labeled and indicated by dashes.
insertion in the presence of Mn\(^{2+}\) in solution (Table 1) and in crystalllo (Supplementary Fig. 2a) is higher compared to that observed with Mg\(^{2+}\), whereas for pol \(\beta\), the rate is not dependent on the identity of these two metals. Unlike pol \(\beta\), catalysis in crystalllo is observed in the absence of a product Mg\(^{2+}\) for pol \(\mu\), indicating that Mg\(^{2+}\) is either very transient, precluding its observation, or not essential for catalysis.

Recent computational studies of pol \(\beta\) have shown that Mg\(^{2+}\) occupancy of the product metal site does not affect the reaction barrier for the forward nucleotidyl transfer reaction, but blocks the reverse reaction. On the other hand, the presence of the catalytic Mg\(^{2+}\) was required for the pol \(\beta\) reverse reaction to occur. In pol \(\mu\), by analogy to pol \(\beta\), loss of the catalytic metal in the Mg\(^{2+}\)-dependent reaction might be expected to block the reverse reaction. In the Mn\(^{2+}\)-dependent reaction, the retention of Mn\(_e\) would be expected to enable the reverse reaction. Nevertheless, in this situation, the presence of Mn\(_p\) would be expected to block the reverse reaction and stabilize the PP\(_i\) product, thereby promoting the forward reaction.

A significant thio-effect for insertion of the S\(_p\)-isomer of TTP (\(\alpha\)S) in the presence of magnesium provides evidence that chemistry is rate limiting during nucleotide insertion. Historically, a modest thio-effect for polymerase-dependent nucleotide insertion suggested that a non-chemical change limits nucleotide insertion rather than chemistry. Since the theoretical magnitude of the thio-effect on chemistry is uncertain, definitive interpretations concerning modest elemental effects are precluded. For pol \(\mu\), however, the strong thio-effect for insertion of the S\(_p\)-isomer of TTP(\(\alpha\)S) in the presence of magnesium indicates that chemistry is rate limiting during nucleotide insertion. Since a strong thio-effect (~24) was observed for pol \(\mu\) with magnesium (Table 1), the loss of this thio-effect with manganese is consistent with the observed increase in the rate of manganese-dependent nucleotide insertion, indicating that as the rate of the chemical step is increased (i.e., in the presence of manganese), a slower non-chemical step becomes rate limiting.

While it may be tempting to correlate the presence of a third or product metal with rate limiting chemistry events, two lines of evidence suggest the product-associated metal is not directly involved in chemistry. A critical aspect of this conclusion arises from the fact that the product-associated metal is not observed until after insertion has occurred (i.e., post-chemistry). Second, it is generally accepted that the strong preference for the S\(_p\)-isomer of the incoming nucleotide is due to its metal-binding characteristics. Accordingly, polymerase insertion studies employing a mixture of R\(_p\)- and S\(_p\)-isomers revealed that only the S\(_p\)-isomer was inserted. Structural studies have confirmed that the pro-R\(_p\)-oxygen coordinates active site metals while a water molecule coordinates the pro-S\(_p\)-oxygen in X- and some Y-family DNA polymerases. Importantly, since the pro-S\(_p\)-oxygen coordinates the product manganese, sulfur substitution at this oxygen would deter binding of the product-associated manganese. This is because manganese is considered thio-phobic preferring to coordinate oxygen. Additionally, the longer P–S bond and larger van der Waals radius of sulfur would preclude product manganese binding. Thus, if the product-associated manganese was directly involved in rate limiting chemistry events, the thio-effect should be much larger than the weak effect observed here.

Pyrophosphate undergoes conformational dynamics during the pol \(\mu\) catalytic cycle. The PP\(_i\) product is observed to rotate ~90° in the 60 min soak with Mg\(^{2+}\) (Fig. 3c, d). The latter conformation is stabilized by interactions with Arg320 and O3' of the newly formed primer terminus. Increased dynamics in Py of the PP\(_i\),
Table 1 Summary of single turnover kinetic parameters

| Metal       | TTP (αX)² | k<sub>pol</sub> (s⁻¹) | K<sub>d</sub> (µM) | k<sub>pol</sub>/K<sub>d</sub> (µM⁻¹·s⁻¹) | k<sub>pol(0)</sub>/k<sub>pol(5)</sub> |
|-------------|-----------|------------------------|-------------------|----------------------------------------|----------------------------------|
| Mn²⁺       | O         | 6.0 (0.2)²             | 192 (21)          | 0.031 (0.004)                          | 24                               |
|             | S         | 0.25 (0.01)            | 10.5 (1.7)        | 0.024 (0.004)                          |                                  |
| Mg²⁺       | O         | 81 (7)                 | 54 (18)           | 1.5 (0.5)                              |                                  |
|             | S         | 26 (1)                 | 27 (5)            | 1.0 (0.2)                              |                                  |

¹Identity of the atom at the pro-S position of Pe (TTP)
²Number in parentheses refers to the SE derived from a fit of dNTP dependencies on the observed single-exponential rates to a hyperbolic equation

Table 2 Comparison of structurally equivalent residues involved in catalysis in pols β and μ

| Pol β Function | Pol μ Function |
|---------------|---------------|
| Ser180 Binds β/γ phosphate | Gly320 Binds β phosphate |
| Arg183 Binds β phosphate | Arg323 Binds β phosphate |
| Arg149 Binds γ phosphate | Lys325 Binds γ phosphate |
| Gly189 Binds γ phosphate | His329 Binds γ phosphate |
| Asp190 Metal coordination | Asp330 Metal coordination |
| Asp192 Metal coordination | Asp332 Metal coordination |
| Arg254 Stabilizes Asp256 and PT<sup>c</sup> | Arg416 Stabilizes Asp418 and PT |
| Asp256 Coordinates PT and Me<sup>b</sup> | Asp418 Coordinates PT and Me |
| Phc272 Modules Asp258/Asp192 | Trp434 Stabilizes sugar of PT |
| Asp276 Stabilizes NT<sup>c</sup> base | Lys438 Stabilizes NT base |

<sup>a</sup>PT Primer terminus
<sup>b</sup>Me<sup>c</sup>, Catalytic metal
<sup>c</sup>NT Incoming nucleotide

product is observed throughout the Mn²⁺-mediated reaction (Fig. 5a, c). This most likely represents conformational flexibility of PP<sub>1</sub> that could not be accurately modeled. These additional conformations were therefore omitted from the models. PP<sub>1</sub> dissociates first during PP<sub>1</sub> release from the pol μ active site, with retention of the Mg²⁺ nucleotide-binding metal (Supplementary Fig. 3). Density for PP<sub>1</sub> progressively weakens during the reaction and is almost completely lost in the 270 and 960 min soaks with Mg²⁺ (Fig. 3 and Supplementary Figs. 2a and 3a). Manganese promotes product retention in the active site, as PP<sub>1</sub> is still present in the 60 min soak (Supplementary Fig. 2c, d). In the 960 min soak (Supplementary Fig. 4), a glycolate molecule (potential cryo-contaminant) is modeled into density corresponding to the PP<sub>1</sub>-binding site<sup>41</sup>. The presence of PP<sub>1</sub> at this site cannot be excluded, however, the shifted location of the observed density suggests the presence of a non-canonical ligand or PP<sub>1</sub> conformation.

The lack of large-scale conformational changes in pol μ or its substrates enables its role in NHEJ. Large-scale conformational changes could impede its biological functions. A rigid platform for synthesis could support pol μ to more effectively utilize variable DNA structures required in repair, since NHEJ processes DNA structures that have dynamic DNA ends (i.e., non-complementary) or DNA strands (i.e., non-contiguous). Curiously, the relatively weak-binding affinity for dNTPs suggests that pol μ could be limited by nucleotide pool concentrations or utilize NTPs that are often found at higher cellular concentrations. Interestingly, a role of the product metal in retarding the reverse reaction could facilitate downstream reactions or interactions with enzymes such as DNA ligase IV.

Methods

Protein expression and purification. Truncated human wild-type pol μ was overexpressed from the pGEXM vector in BL21(DE3)CodonPlus-RIL cells overnight at 16 °C and purified as follows<sup>25</sup>. Briefly, cells were lysed by sonication in lysis buffer (25 mM Tris, pH 8 (25 °C), 500 mM NaCl, 5% glycerol, 1 mM DTT) and purified in batch on glutathione Sepharose 4B resin (GE Healthcare). Pol μ was then purified by size-exclusion chromatography on Superdex 200 26/60 in gel filtration buffer (25 mM Tris, pH 8 (25 °C), 100 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA).
EDTA was added to the gel filtration buffer to remove contaminating divalent metals. The combined fractions (20–30 ml) were then dialyzed against the final buffer (25 mM Tris, pH 8.0 (25 °C), 100 mM NaCl, 5% glycerol, 1 mM DTT), concentrated to ~11 mg ml⁻¹, and flash frozen in liquid nitrogen.

**DNA preparation.** Purified oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Short oligonucleotides (i.e., 4-mers) were desalted and used with further purification. A 9-mer template oligonucleotide (5′-GGGATACG-3′) was annealed with a 4-mer upstream (5′-GGTA-3′) oligonucleotide and a 5′-phosphorylated downstream 4-mer (5′-GGGC-3′) oligonucleotide in a 1:1:1 ratio to create a duplex DNA with a single-nucleotide gap. Oligonucleotides were dissolved in 100 mM Tris-HCl, pH 7.5, heated to 95 °C for 5 min, and then cooled to 4°C at a rate of 1 °C min⁻¹ and kept on ice until use. For kinetic assays, a 29-mer template oligonucleotide (5′-CAGTCTGACTGCAGGCTTGGTCCCT-5′) was annealed with 14-mer upstream (5′-GC(T)3GA(T)CAGTGACTGACTG-3′) and 14-mer downstream (5′-GCCGGGACGGACGGAG-3′) oligonucleotides to create a single-nucleotide gap (template nucleotide underlined). Oligonucleotides were resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and mixed in a 1:2:1:2 ratio (template:downstream:upstream), then annealed as described above.

**Time-lapse crystallography.** Binary complex crystals of pol μ with a templating adenine in a 1-nucleotide gapped DNA were grown at 4 °C, by mixing pol μ, CaCl₂, but instead of CaCl₂, containing 10 mM MgCl₂ or 10 mM MnCl₂ for (previously truncated to improve crystallizability25), and are on the border of the Ramachandran analysis determined 99.7% of residues lie in allowed regions and at of interest and performing simulated annealing with harmonic restraints. PyMol45, and all omit electron density maps were generated by deleting the regions |ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/s41467-017-00271-7|

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**Gap-filling kinetic assays.** Single-turnover kinetic assays were performed on a Kinetek RQF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX), to directly measure the rate of the first insertion (k₁obs) and the apparent equilibrium nucleotide dissociation constant (Kₐapp). Assays were performed in a buffer containing 50 mM Tris-HCl, pH 7.4 (37 °C), 100 mM KCl, 10% glycerol, 100 µg ml⁻¹ bovine serum albumin, 1 mM diithiothreitol, and 0.1 mM EDTA. Briefly, 1 mM pol μ was pre-incubated with 100 nM single-nucleotide gapped DNA substrate and rapidly mixed with varying concentrations of metal-TTP (1:1, v/v). The concentration of Mg²⁺ or Mn²⁺ was adjusted to account for equimolar metal binding by TTP, so at least 10 nM Mg²⁺ or 1 mM Mn²⁺ free metal was present. Reactions were quenched with 0.25 M EDTA and mixed with an equal volume of formamide dye. Products were separated on 18% denaturing gels and quantified using a Typhoon phosphorimager and Imagequant software. Time courses were fit to a single exponential, and a secondary plot of the dNTP dependencies on the observed single-exponential rates were fit to a hyperbolic equation22.

**Data availability.** Coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank under the accession codes 5TXK, 5TXZ, 5TYR, 5TYT, 5TYD, 5TYG, 5TYU, 5TYV, 5TYW, 5TYX, 5TYY, and 5TYZ. Other data that support the findings of this study are available from the corresponding author on reasonable request.
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Author contributions
J.A.J., W.A.B., L.C.P., and S.H.W. designed the project; J.A.J. performed the crystallography. D.D.S. and W.A.B. performed the kinetic experiments; J.A.J., W.A.B., L.C.P., D.D.S., A.F.M., J.M.K. K.B., T.A.K., and S.H.W. analyzed the data; J.A.J., W.A.B., L.C.P., and S.H.W. wrote the paper; S.H.W. directed and supervised the research.

Additional information
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