Quality control mechanisms exclude incorrect polymerases from the eukaryotic replication fork

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The eukaryotic genome is primarily replicated by two DNA polymerases, Pol ε and Pol δ, that function on the leading and lagging strands, respectively. Previous studies have established recruitment mechanisms whereby Cdc45-Mcm2-7-GINS (CMG) helicase binds Pol ε and tethers it to the leading strand, and PCNA (proliferating cell nuclear antigen) binds tightly to Pol δ and recruits it to the lagging strand. The current report identifies quality control mechanisms that exclude the improper polymerase from a particular strand. We find that the replication factor C (RFC) clamp loader specifically inhibits Pol ε on the lagging strand, and CMG protects Pol δ against RFC inhibition on the leading strand. Previous studies show that Pol δ is slow and distributive with CMG on the leading strand. However, Saccharomyces cerevisiae Pol δ–PCNA is a rapid and processive enzyme, suggesting that CMG may bind and alter Pol δ activity or position it on the lagging strand. Measurements of polymerase binding to CMG demonstrate Pol ε binds CMG with a Kₐₐ value of 12 nM, but Pol δ binding CMG is undetectable. Pol δ, like bacterial replicases, undergoes collision release upon completing replication, and we propose Pol δ–PCNA collides with the slower CMG, and in the absence of a stabilizing Pol δ–CMG interaction, the collision release process is triggered, ejecting Pol δ on the leading strand. Hence, by eviction of incorrect polymerases at the fork, the clamp machinery directs quality control on the lagging strand and CMG enforces quality control on the leading strand.

replisome | replication | clamp loader | PCNA | DNA polymerase

Duplication of genetic material is performed by a dynamic interplay of numerous different proteins, collectively referred to as the replisome, that orchestrate their actions to accomplish efficient, high-fidelity replication of both the leading and lagging strands (1–3). Eukaryotes possess several replisome factors that have no homologs in bacteria, and also require two different DNA polymerases for bulk leading and lagging strand synthesis, and Escherichia coli and its phases use identical copies of a DNA polymerase for both strands of the duplex (4). Thus far, the evolutionary purpose behind the additional complexity in eukaryotes is unclear.

At the heart of the eukaryotic replisome is an 11-subunit helicase complex referred to as CMG (Cdc45-Mcm2-7-GINS) (5–7). Eukaryotes use three different multisubunit B-family DNA polymerases (Pol) for replication. Pol ε and Pol δ copy the bulk of the genome, and Pol α–primase generates hybrid RNA–DNA primers of about 25 nucleotides and, unlike Pol ε and Pol δ, it lacks 3′-5′ exonuclease (proofreading) activity. The replication factor C (RFC) clamp loader is used to load PCNA (proliferating cell nuclear antigen) clamps onto primed sites that envelope the replicative polymerases with high processivity (8). The replication protein A (RPA) heterotrimer binds and protects the single-strand (ss) DNA of the lagging strand against nucleases, and helps melt secondary structure in ssDNA. Reconstitution of the core eukaryotic replisome using all three replicative DNA polymerases has recently been accomplished with pure recombinant proteins in the S. cerevisiae system (9–11). These in vitro studies revealed that Pol ε binds directly to CMG, forming a CMGE complex (a complex of CMG bound to Pol epsilon), and this recruits Pol ε to PCNA on the leading strand for efficient extension. However, our earlier studies made the perplexing observation that Pol ε was incapable of lagging strand synthesis even in the absence of Pol δ (9, 10). We and others have shown that S. cerevisiae Pol δ–PCNA is rapid, over 100 bp/s, and its processivity varies depending on ionic strength (12, 13). The intracellular ionic strength of S. cerevisiae is unknown, but at ionic strength under 70 mM, Pol δ–PCNA is highly processive for over 5 kb (13). Surprisingly, under these highly processive conditions Pol δ–PCNA exhibited poor and distributive activity with CMG on the leading strand (10). These paradoxical findings suggest that, in addition to recruitment mechanisms that place the proper polymerases on their respective strands, there also exist quality control mechanisms that remove improperly placed DNA polymerases on the wrong strand. These quality control processes were unanticipated from bacterial studies, possibly because bacteria generally use identical copies of DNA polymerases for both strands.

The recruitment processes that target Pol ε and Pol δ to their respective strands in vitro support conclusions from numerous yeast genetics experiments in S. cerevisiae and Schizosaccharomyces pombe (14–18). However, the existence of quality control reactions that excluded these same polymerases from their opposite strand was unanticipated from the cell biology and genetics. Hence, the current report examines the mechanisms that underlie the quality control reactions that eliminate Pol ε from the lagging strand, and that largely prevents Pol δ synthesis on the leading strand, as informed from earlier studies of eukaryotic replisome reconstitution reactions (9–11).

Results and Discussion

RFC Underlies Quality Control of Pol ε on the Lagging Strand. We have shown previously that a reconstituted 31 protein system consisting of CMG, Pol ε, Pol δ, Pol α, RFC, PCNA, and RPA, a total of 31 distinct polypeptides, recapitulates the cellular findings that Pol ε

Significance

DNA replication is a central life process and is performed by numerous proteins that orchestrate their actions to separate the strands of duplex DNA and produce new copies of the genome for cell division. While the antiparallel architecture of DNA is elegant in its simplicity, replication of DNA still holds many mysteries. For example, many essential replication proteins still have unknown functions. In eukaryotes the two DNA strands are duplicated by different DNA polymerases. The mechanism by which these different polymerases target to their respective strands is understood. This report examines the mechanisms that eject incorrect polymerases when they associate with the wrong strand.

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and Pol δ are mainly active only on their respective leading and lagging strands (9–11). Although we have determined the recruitment processes that target the polymerases to their sites of action, we also noticed that Pol δ is largely inactive on the leading strand in the absence of added Pol ε, and that Pol ε is mysteriously inactive on the lagging strand, even in the absence of Pol δ (10). Hence, besides recruitment processes, there are also quality control processes that clear polymerases from the incorrect strand. Polymerase quality control reactions are not needed for bacterial replisomes, because identical polymerases extend both the leading and lagging strand.

While investigating these eukaryotic quality control processes we noticed that RFC sometimes inhibits Pol ε, a paradoxical observation because Pol ε has been shown to be stimulated by PCNA (12). To understand this perplexing phenomenon we titrated RFC into reactions containing PCNA and primed ssDNA, and compared results using either Pol ε or Pol δ (Fig. 1). Interestingly, Pol ε was initially stimulated by RFC/PCNA at low concentrations of these reagents, but was drastically inhibited by even a modest increase in RFC (Fig. 1A). In contrast, Pol δ is not significantly affected at relatively high concentrations of RFC (Fig. 1B). Addition of extra RFC after initiating a moving Pol ε–PCNA also inhibits synthesis, demonstrating that RFC can stop Pol ε during ongoing synthesis (Fig. S1). RFC also inhibited Pol α on primed ssDNA but to a lesser degree (Fig. S2), similar to previous work (19). We explore Pol α later in this report.

To test whether the basis of inhibition of Pol ε by RFC might be direct competition for the primed site, we determined the equilibrium binding constant (K\textsubscript{d}) for both RFC and Pol ε to primed DNA using fluorescence anisotropy (Fig. 2A). RFC and Pol ε were individually titrated into reactions containing a fluorescently tagged primed DNA, and the fluorescence anisotropy of the DNA was determined. Measuring the change in fluorescence anisotropy at different concentrations of RFC or Pol ε provided K\textsubscript{d} values of 5.17 ± 0.5 and 8.22 ± 0.7 nM for RFC and Pol ε, respectively (Fig. 2A). Use of a fluorescently tagged dsDNA substrate in similar experiments gave much weaker binding (82.4 ± 12.9 and 80.5 ± 16.5 nM for RFC and Pol ε, respectively), as did use of an unprimed ssDNA substrate (42.4 ± 4.9 and 62.0 ± 9.0 nM for RFC and Pol ε, respectively; Fig. 2B, Fig. S3). These values are consistent with an earlier qualitative study that observed a higher affinity of RFC for a primed site compared with ssDNA and dsDNA substrates (20). The fact that RFC and Pol ε have similar binding constants for primed DNA suggests that inhibition of Pol ε by RFC may be due to a simple competition for the primer terminus.

To determine whether competition between RFC and Pol ε is physiologically plausible, we estimated the intracellular concentrations of RFC and Pol ε. Intracellular concentrations of Pol ε and/or RFC can be approximated from proteomics data in which a sequence encoding a TapTag was cloned onto the end of each of the genes in the genome (21). Taking the average copy number of all subunits in each protein and assuming a nuclear volume of 2.9 fL (22), we calculate the nuclear concentrations of RFC and Pol ε to be ~200 and 1.1 μM, respectively. Using the K\textsubscript{d} parameters measured herein to estimate competition for primed sites in the yeast genome (assuming one primed site per origin), RFC would greatly outcompete Pol ε at primed sites (Fig. S4; Supporting Information). Pol δ displayed undetectable affinity for the primed site (Fig. S5), but as shown later in this report, Pol δ circumvents competitive inhibition with RFC through tight binding to PCNA compared with Pol ε.

We ruled out additional differences between leading and lagging strand synthesis that may further explain the inability of Pol ε to extend Okazaki fragments. Specifically, we show that the inability of Pol ε to operate on the lagging strand does not arise from RPA inhibition or through initiation on short primers like those synthesized by Pol α (Fig. S6; Supporting Information). Because RPA has been shown to help direct RFC to the primer terminus (23), we also considered the possibility that inhibition of Pol ε by RFC required an interaction with RPA, however, we observe inhibition of Pol ε by RFC in the presence of E. coli SSB as well (Fig. S7). We also examined the effect of CMG on Pol ε activity on RPA-coated PCNA-primed ssDNA, to determine whether CMG may directly inhibit Pol ε. Unexpectedly, the result showed instead that CMG stimulates the rate of Pol ε on an RPA-coated PCNA-primed ssDNA (Fig. S8), from which we infer that formation of the previously documented CMGE complex (11, 24) enhances Pol ε enzymatic activity even in the absence of helicase activity.

CMG Prevents RFC Inhibition of Pol ε on the Leading Strand. RFC is a potent inhibitor of Pol ε on a primed site as demonstrated in Fig. 1, yet Pol ε must retain function on the leading strand. How can Pol ε function on the leading strand when inhibitory levels of RFC are present in the cell? To address this question, RFC was titrated into CMGE-dependent leading strand reactions on a 3-kb linear forked DNA primed with a 5′–3′-oligonucleotide (scheme; Fig. 3A). Surprisingly, Pol ε was not inhibited by RFC even at 80-nM RFC (Fig. 3B), whereas only 10-nM RFC strongly inhibited Pol ε in the lagging strand model (Fig. 1A).

CMG is known to bind Pol ε, and we presume CMG is the entity that protects Pol ε from RFC inhibition on the leading strand. To determine whether the affinity of Pol ε for CMG is sufficiently tight to form a complex in the cell, we determined the equilibrium dissociation constant (K\textsubscript{d}) of Pol ε binding to CMG (Fig. 4A and B). We labeled CMG with Cy5-NHS and measured equilibrium binding isotherms using microscale thermophoresis (MST;
Methods) (Fig. 4A). Holding the CMG concentration at 4 nM, we titrated Pol ε into the reactions. As a control, CMG showed no aggregation or capillary adsorption (Fig. S9). An infrared laser was applied at t = 0, and we observed a depletion of fluorescence signal from the irradiated region due to the thermophoretic effect, followed by a return of fluorescence to the heated region at a rate that was directly dependent on Pol ε concentration (Fig. 4B). Plotting Pol ε concentration versus the change in Fnorm, defined as Fcold/Fhot or the ratio of the fluorescence intensity before and after application of the laser, we observed a binding isotherm for CMG binding to Pol ε, revealing a tight binding of CMG to Pol ε with an apparent dissociation constant (Kdapp) of 12.7 ± 2.0 nM (Fig. 4C). As described above, we estimate the nuclear concentration of Pol ε to be ~1.1 μM, and similar calculations of CMG suggest about 4.7 μM CMG in the nucleus (Supporting Information). Assuming these intracellular concentrations and the Kd values determined here, Pol ε is in complex with CMG in the cell.

Pol δ–PCNA Provides a Second Quality Control Process that Prevents Pol ε Action on the Lagging Strand. How does Pol δ overcome inhibition by RFC? The affinity of Pol δ to a primed site was too weak to measure, as we did not observe Pol δ binding to DNA in our anisotropy experiments (Fig. S5). Both Pol ε and Pol δ are stimulated by PCNA (Fig. S10). However, if Pol δ were to bind the PCNA more tightly than Pol ε, it could drive the RFC clamp loading reaction forward rather than suffer inhibition like Pol ε. Indeed, we have shown previously that in a mixture of Pol ε and δ, Pol δ preferentially binds to PCNA-primed DNA, competing with and preventing Pol ε from function on a PCNA-primed template (9).

In Fig. 5 we analyzed the Kd values of either Pol δ or Pol ε to PCNA using MST and Cy5-labeled PCNA. The fluorescently labeled PCNA was first loaded onto a synthetic primed site using RFC and ATP, along with an excess of primed DNA to ensure only one clamp was loaded per DNA molecule. Titration of Pol ε into the preformed PCNA–DNA yields data that fit a Kd value of 326 ± 102 nM between Pol ε and primer-loaded PCNA (Fig. 5A). Using a similar approach, we calculated the affinity of Pol δ for PCNA-DNA to be 13.7 ± 1.3 (Fig. 5B). Thus, Pol δ binds PCNA–DNA 24-fold more avidly than Pol ε. These measurements reveal a second quality control process that prevents Pol ε from acting on the lagging strand. Specifically, Pol δ outcompetes Pol ε for PCNA on DNA. Interestingly, our earlier study showed that Pol ε function with PCNA on the leading strand with CMG was impervious to takeover by Pol δ, revealing that CMG protected Pol ε from Pol δ, much as CMG protects Pol ε from RFC (9).

Quality Control of Pol δ on the Leading Strand. One simple quality control process to clear Pol δ from the leading strand would be if Pol δ is distributive and often spontaneously dissociates from DNA. This would provide ample opportunity for the CMG-stabilized Pol ε to take over the leading 3' terminus. In fact, human Pol δ is known to be distributive with PCNA (25), and barring stability by another protein, quality control on the leading strand may be explained by spontaneous dissociation in humans. We note that different processivity values of S. cerevisiae Pol δ–PCNA are reported, which probably depend on the substantially different ionic strengths used in the studies, but could also possibly be due to different methods of protein preparation (12, 13). It is important to note that the intracellular salt in yeast is not yet identified, nor has the intracellular ionic strength of S. cerevisiae been determined. The buffer used here contains 85 mM salt, yet Pol δ–PCNA is processive for over 5 kb (13). However, if the intracellular ionic strength is sufficient to destabilize Pol δ–PCNA, then spontaneous dissociation of Pol δ from DNA combined with CMG induced stability of Pol ε on the leading strand could explain leading strand quality control.

Insight into a quality control process that removes a tightly bound and highly processive polymerase from DNA may be gleaned from bacterial studies. We initially identified a reaction within the E. coli Pol III replicase in which it is highly processive during synthesis, but upon completing a template, Pol III ejects from DNA (26, 27). Ejection is not immediate because Pol III can still diffuse short distances over dsDNA upon completion (28), but Pol III dissociation occurs within 1–2 s leaving the clamp behind on DNA (25). In bacterial replication this “collision release” reaction is one of the pathways that explain how a highly processive polymerase can

Fig. 3. RFC does not inhibit Pol ε during function with CMG in leading strand synthesis. (A) Schematic of the reaction. RFC is titrated into CMG-directed Pol ε (leading strand) reactions on a 3-kb linear substrate ligated to a forked junction primed with a 5'-2P labeled primer. (B) Alkaline agarose gel of extension products in the presence of the indicated RFC concentrations. Reactions were stopped at the indicated times. “FL” indicates the full length (3.2 kb) product.

Fig. 4. CMG tightly binds Pol ε, but does not bind Pol δ. (A) Schematic of MST assay. Pol ε or Pol δ is mixed with fluorescently labeled CMG. A localized IR beam creates a temperature gradient, and thermophoresis causes depletion of molecules from the observation volume. During equilibrium, some molecules return toward the heat gradient. (B) MST traces for CMG binding to Pol ε. Arrow indicates increasing Pol ε concentration. Fcold and Fhot are depicted as blue and red regions. (C) Binding isotherm for CMG and Pol ε obtained from plotting ∆Fnorm (the change in the Fcold/Fhot ratio) vs. Pol ε concentration. The fitted Kdapp value is shown. (D) MST traces and (E) binding curve for CMG binding to Pol δ. Data are presented as mean ± SD.
rapidity recycle from the end of an Okazaki fragment to a new primed site during synthesis of numerous lagging strand fragments (26, 27). Unexpectedly, we discovered that \textit{S. cerevisiae} Pol δ–PCNA also contains a collision release mechanism, similar to that originally observed in \textit{E. coli} Pol III (13). We presume Pol δ–PCNA collision release takes a few seconds, like the \textit{E. coli} replicase, because elegant rapid reaction studies by the Burgers group have shown that Pol δ remains with PCNA for the few seconds it requires to function with Fen1 in removal of RNA and to perform gap fill-in during Okazaki fragment repair (29). Although collision release may be useful to lagging strand synthesis in any organism, we propose that the collision release mechanism of Pol δ–PCNA is a leading strand quality control mechanism in eukaryotes.

Although the rapid speed (>100 bp/s) and high processivity of Pol δ–PCNA makes it an attractive candidate for the job of continuous synthesis on the leading strand, Pol δ–PCNA function with CMG displays a slow rate (1–2 bp/s) and is distributive on the leading strand (9, 13). The rapid rate of an unimpeded Pol δ–PCNA implies that a leading strand Pol δ–PCNA would be in constant contact with CMG which encircles the leading strand and slowly advances the forked junction at a measured rate in vivo of 32 bp/s (30). Thus, most of the time Pol δ–PCNA would be waiting behind CMG for a new unwound leading strand template to be produced. In this condition, similar to completing a template, it seems likely that Pol δ–PCNA would undergo collision release when it “collides” with CMG, upon which Pol δ would depart from DNA leaving PCNA behind. Alternatively, there is a report that Pol δ functions as the normal polymerase on the leading strand instead of Pol ε (31), and therefore a physical interaction of CMG with Pol δ might stabilize Pol δ–PCNA and retard the collision release process.

To determine whether CMG indeed interacts with Pol δ, we examined binding of CMG to Pol δ via the MST technique (Fig. 4D). Interestingly, titration of Pol δ into reactions with CMG reveals no detectable interaction between Pol δ and CMG (Fig. 4D and E). Thus, if there were an interaction between CMG and Pol δ, the affinity would be at least 3 orders of magnitude weaker than the interaction between CMG and Pol ε. Therefore, CMG does not bind and stabilize Pol δ–PCNA. We therefore propose that Pol δ–PCNA undergoes collision release upon the encounter with CMG, accounting for the observed slow and distributive action of Pol δ–PCNA on the leading strand (9).

Important to any discussion of Pol δ on the lagging strand is the fact that the N-half of the Polε gene encoding the active polymerase within Pol ε can be deleted and cells retain viability, although they are severely compromised in growth (32). It is proposed that under these conditions Pol δ takes over leading strand synthesis (32). Our results using Pol δ in the absence of Pol ε demonstrate that Pol δ can, in fact, function on the leading strand, but that its rate and processivity are diminished probably due to the quality control process described above. However, the residual activity of Pol δ on the leading strand may very well be capable of completing replication in cells that are compromised in Pol ε activity. As mentioned above, one recent report implies that Pol δ functions on both strands during normal replication (31). However, active site mutants of Pol ε are not viable, demonstrating that Pol ε is used during normal replication (33). We presume, given the weight of the conclusions from genetics and biochemistry studies, that Pol ε is the normal leading strand polymerase, and that other polymerases can take over when it is compromised (as recently discussed in ref. 34).

Lastly we determined whether Pol δ could still function on the lagging strand at very high RFC concentration while acting in concert with the proteins of the eukaryotic replisome holoenzyme. Hence, we examined the effect of RFC and Pol δ on Okazaki fragment synthesis in replisome reconstitution assays containing CMG, PCNA, RFC, RPA, and Pol α, Pol ε, and Pol δ using a 3-kb duplex to which a synthetic forked junction is ligated. The 3-kb replication fork substrate contains no dG residues on one strand and thus no dC on the other, enabling the specific labeling of the lagging strand using \textsuperscript{32}P-dGTP (Fig. 6A). Initiation of synthesis on the lagging strand requires the primase activity of Pol α, and therefore we first examined reactions using CMG and Pol α with no other polymerases present, and titrated RFC. The results of Fig. 6B show that titration of RFC into Pol α/CMG/PCNA reactions has an inhibitory effect on Pol ε–primase, and this is described in more detail below. In Fig. 6B, reactions containing Pols α and ε, but no Pol δ demonstrate that Pol ε is incapable of Okazaki fragment extension, as shown in our earlier studies (10). The last three lanes that include Pols α, ε, and δ in reactions containing 80 nM RFC show that Pol δ provides robust lagging strand synthesis, filling in the primed lagging strand fragments even at a high concentration of RFC (Fig. 6B).

**Quality Control of the DNA Polymerase of Pol α–Primase**. Pol α–primase contains both priming and DNA polymerase activities, and left unchecked, the polymerase activity of Pol α–primase has been demonstrated to function with CMG to replicate both the leading and lagging strands (9, 10). Pol α–primase was earlier shown to function with the SV40 T-antigen helicase to synthesize both the leading and lagging strands of the SV40 genome (35). Limiting Pol α–primase function is important because the enzyme lacks a proofreading exonuclease, and thus has lower fidelity than either Pol ε or Pol δ (reviewed in ref. 36). Pol δ is known to switch with Pol α–primase in the SV40 system (37). Our earlier work revealed that both Pol ε and Pol δ were capable of rapidly taking over a primed site from Pol α–primase, regardless of which strand Pol α–primase was located on.
Polymerase switching with Pol α–primase will provide a quality control process to limit the polymerase activity of Pol α–primase, depending on how quickly the polymerase switch occurs. Considering that primed sites are acted upon by RFC/PCNA before either Pol ε or Pol δ can become processive, we examined whether RFC provides an additional level of quality control that limits the polymerase activity of Pol α–primase.

In the experiment presented in Fig. 6, which examines the effect of RFC on leading strand synthesis in the reconstituted replication system, the first three lanes show reaction products using Pol α with no other polymerases present at different concentrations of RFC. The results show that titration of RFC inhibits Pol α–primase. The reduction in Okazaki fragment length observed in the RFC titration is likely due to competition of RFC with the DNA polymerase of Pol α for primer termini, because in the absence of primer synthesis RFC would have no other substrate to bind. This conclusion is supported by the experiment in Fig. S2 that directly demonstrates RFC-mediated inhibition of the DNA polymerase of Pol α–primase in extension of DNA oligonucleotide primed φX174 ssDNA. Thus, RFC inhibits Pol α–DNA synthesis. The result does not exclude the possibility that RFC also prevents primer synthesis by Pol α–primase. However, the last three lanes in Fig. 6 that include Pol α, ε, and δ show robust lagging strand synthesis, and this indicates that Pol α priming activity is not inhibited by RFC, as Pol δ requires primed sites for function.

It has long been known that eukaryotes require three DNA polymerases for chromosomal replication, yet only recently has the stand assignments of each polymerase been identified, primarily based on in vivo genetics experiments tracking unique mutator phenotypes of Pol ε and Pol δ in budding and fission yeast (14–17). These studies place Pol ε on the leading strand and Pol δ on the lagging strand (34). Biochemical reconstitution of a functional leading–lagging strand eukaryotic replisome from pure proteins has been accomplished recently, enabling an independent method to assess the identity of polymerase assignment to specific DNA strands, as well as the mechanisms that underlie these assignments (9, 10). Although polymerase recruitment mechanisms were expected based on bacterial studies, we did not anticipate the existence of quality control mechanisms in eukaryotes that remove polymerases that attach to the “wrong” strand of DNA. A model of quality control at the replication fork is presented in Fig. 7. Although we have explored these processes in this report, there may be yet other quality control mechanisms that further enforce specific polymerase utilization on particular DNA strands to be discovered in future studies.

**Methods**

**Protein and DNA Constructs.** Proteins were purified as previously described (9). The Pol ε and Pol δ used in the fluorescence anisotropy and MST experiments were made enoxonucleases deficient by site-directed mutagenesis. Proteins used as probes for MST experiments were nonspecifically labeled with Cy5-NHS (GE Healthcare). DNA replication templates were prepared as previously described (9), and the DNA substrates used in anisotropy experiments were ordered by Integrated DNA Technologies (IDT). See Supporting Information for full details including DNA sequences.

**Replication Assays.** Reaction volume reactions were 25 μL and contained 25 mM Tris-OAc pH 7.5, 5% (vol/vol) glycerol, 40 μg/mL BSA, 5 mM DTT, 10 mM Mg-OAc, 50 mM K glutamate, 0.1 mM EDTA, 60 μM each dTTP and dATP, 20 μM each dCTP and dGTP, 5 mM ATP, 200 μM each CTp, UTP, GTP, and 10 μCi of the cognate nucleotide for leading strand (α-32P-dCTP) or lagging strand (δ-32P-dGTP) reactions unless otherwise noted. All protein titrations were balanced for incoming salt. See Supporting Information for full details.

**Fluorescence Anisotropy.** Anisotropy reactions were 20 μL in 25 mM Tris-OAc pH 7.5, 5% (vol/vol) glycerol, 40 μg/mL BSA, 5 mM DTT, 1 mM EDTA, 25 mM K glutamate, and 1 mM probe DNA with a terminal fluorescein. Titrations of Pol ε or Pol δ were scanned on a Biotek Neo 2 384-well plate reader (Biotek Instruments). Binding curves were fit to a single-site binding equation using the Origin (OriginLab) software. See Supporting Information for full details.

**Microscale Thermophoresis.** MST experiments were performed in 25 mM Tris-HCl pH 7.5, 100 mM K glutamate, 10% (vol/vol) glycerol, 5 mM DTT, 1 mM EDTA, and 0.05% Tween 20; 4 nM CMG-Cy5 and 15 nM PCNA were used as probe. For PCNA-binding experiments, PCNA-Cy5 was first loaded onto the primed template with RFC and ATP, followed by titration with Pol ε or Pol δ. Data were fit to simple binding curves using the MO.Affinity software (NanoTemper). See Supporting Information for full details.

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