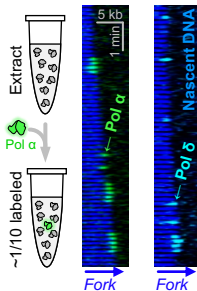
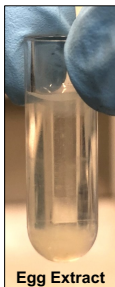
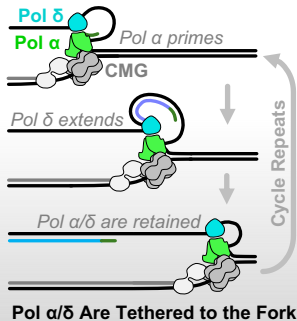
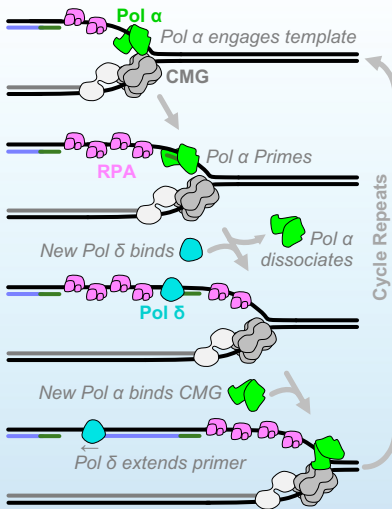


## Old (Trombone) Model



## New Model



## Lagging Strand Synthesis is Blocked



**Leading Strand Synthesis Is Not Affected**

# Metazoan Lagging Strand DNA Synthesis is Distributive

Luke D. Lynch<sup>1</sup>, Jinho Park<sup>2</sup>, Gheorghe Chistol<sup>2,3,4,5,\*</sup>

<sup>1</sup>Biochemistry Department, Stanford School of Medicine, Stanford CA 94305, USA

<sup>2</sup>Chemical and Systems Biology Department, Stanford School of Medicine, Stanford CA 94305, USA

<sup>3</sup>Stanford Cancer Institute, Stanford School of Medicine, Stanford CA 94305, USA

<sup>4</sup>BioX Interdisciplinary Institute, Stanford University, Stanford CA 94305, USA

<sup>5</sup>Lead Contact

\*Correspondence: [chistol@stanford.edu](mailto:chistol@stanford.edu) (G.C.)

Current address: L.D.L.: Department of Biological Chemistry and Molecular Pharmacology, Blavatnik Institute, Harvard Medical School, Boston, MA, USA; Howard Hughes Medical Institute, Boston, MA 02115, USA

## SUMMARY

DNA's anti-parallel structure presents a topological challenge to the replisome, as leading and lagging strands must be synthesized in opposite directions. How lagging strand synthesis is coordinated with leading strand synthesis remains unresolved in eukaryotes. To address this question, we directly visualized the dynamics of lagging strand polymerases Pol  $\alpha$  and Pol  $\delta$  at active replication forks using single-molecule imaging in *Xenopus* nuclear extracts. Surprisingly, we find that neither Pol  $\alpha$  nor Pol  $\delta$  is stably tethered to the replisome. Instead, lagging strand synthesis occurs *distributively* and entails the rapid recruitment of new molecules of Pol  $\alpha$  and Pol  $\delta$  for each Okazaki fragment. Consequently, leading and lagging strand synthesis are not intrinsically coupled. We propose that metazoans solve the problem of anti-parallel replication not by actively coordinating leading and lagging strand synthesis, but instead simply by recruiting new polymerases to synthesize each Okazaki fragment.

## INTRODUCTION

In eukaryotes, Polymerase  $\epsilon$  (Pol  $\epsilon$ ) synthesizes the leading strand continuously, while Polymerase  $\alpha$ -Primase (Pol  $\alpha$ ) and Polymerase  $\delta$  (Pol  $\delta$ ) synthesize lagging strand fragments discontinuously<sup>1-4</sup>. For a typical vertebrate with a 6-billion nucleotide (nt) genome, lagging strand synthesis poses a monumental task, as ~40 million Okazaki fragments (~150 nt/each) must be synthesized before each cell division. First, Pol  $\alpha$  is recruited to the replicative helicase CMG (CDC45•MCM2-7•GINS) and engages the lagging strand template<sup>5</sup>. Second, Pol  $\alpha$  synthesizes a 6-10 ribonucleotide (rNTP) primer<sup>6</sup>, extends it by 20-30 deoxyribonucleotides (dNTPs), then disengages from the template<sup>7,8</sup>. Third, the Proliferating Cell Nuclear Antigen (PCNA) is loaded onto the primer-template junction, promoting the recruitment of Pol  $\delta$ <sup>9</sup>, which extends the primer<sup>10</sup> until colliding with a downstream Okazaki fragment<sup>11</sup>. Finally, the Flap Endo-Nuclease 1 (FEN1) cleaves the RNA primer and neighboring Okazaki fragments are ligated<sup>10</sup>. Primer-template junctions that form transiently during lagging strand synthesis (or persistently during replication stress) activate the kinase ATR (Ataxia-Telangiectasia and Rad3-related) – the master regulator of the S-phase checkpoint<sup>12,13</sup>. ATR phosphorylates the effector kinase CHK1 (Checkpoint Kinase 1) which launches numerous cellular responses to safeguard genome integrity<sup>14</sup>.

Early studies of bacteriophage replication revealed that both the DNA Primase and the lagging strand DNA polymerase are stably tethered to the replisome, enabling it to processively synthesize many consecutive Okazaki fragments<sup>15,16</sup>. To rationalize this, it was proposed that the lagging strand DNA template forms a trombone-like loop to facilitate the coordinated synthesis of leading and lagging strands by a single stable enzyme complex.

Although prokaryotic and eukaryotic replication machineries evolved independently<sup>17</sup>, the trombone model originally proposed for prokaryotes was adopted for eukaryotes. Two recent studies support the trombone model in yeast<sup>18,19</sup> (Figure 1A): an *in vitro* reconstitution study reported that Pol  $\alpha/\delta$  travel with the replisome for tens of minutes – long enough to synthesize hundreds of Okazaki fragments<sup>18</sup>, while another study imaged polymerases in live yeast and concluded that Pol  $\alpha/\delta$  are recycled for multiple Okazaki fragments<sup>19</sup>.

To elucidate the mechanism of lagging strand synthesis in higher eukaryotes, we directly visualized Pol  $\alpha/\delta$  at active forks in *Xenopus* nuclear egg extract, which contains all soluble nuclear proteins and faithfully recapitulates replication<sup>20,21</sup>. Surprisingly, we find that Pol  $\alpha/\delta$  reside at active forks for only 15-30 seconds (whereas Pol  $\alpha$  resides at stalled forks for several minutes). We find that the sudden removal of soluble Pol  $\alpha$  and Pol  $\delta$  from DNA replication reactions abruptly stops the synthesis of new Okazaki fragments. We show that blocking lagging strand synthesis does not impair CMG progression, indicating that leading and lagging strand synthesis are not intrinsically coupled in metazoa. Our data support a *distributive* mechanism of lagging strand

replication, where new copies of Pol  $\alpha/\delta$  must be constantly recruited to the fork to sustain the synthesis of new Okazaki fragments.

## RESULTS

### Neither Pol $\alpha$ Nor Pol $\delta$ Are Stably Tethered to the Replisome During Unperturbed Replication

The defining feature of the trombone model is that lagging strand polymerases are stably tethered to the replisome and should travel with forks for several minutes (Figure 1A). To test this idea, we labeled recombinant Pol  $\alpha/\delta$  (Figure S1A-B) with Alexa-Fluor 647 and monitored the dynamics of Pol  $\alpha^{AF647}$  and Pol  $\delta^{AF647}$  at forks via single-molecule imaging in *Xenopus* egg extracts (Figure 1B).

We flow-stretched  $\lambda$  DNA and tethered it to a functionalized coverslip in a microfluidic flow-cell (Figure 1B). The flow-cell was incubated with *Xenopus* egg extracts that support efficient replication<sup>22</sup>. Extracts were supplemented with FEN1 fused to the fluorescent protein mKikGR (FEN1<sup>mKikGR</sup>), which serves as a marker of lagging strand synthesis<sup>23</sup>.

First, we spiked 20 nM of Pol  $\alpha^{AF647}$  into undepleted extract, which contains ~700 nM of endogenous Pol  $\alpha$ . Pol  $\alpha^{AF647}$  bound to elongating replisomes and resided at forks for ~30 seconds before dissociating (Figure 1C-D, light green). In egg extracts, forks travel at 300-500 nt/min and Okazaki fragments measure 100-500 nt<sup>24</sup>, therefore 30 seconds corresponds to 1-2 Okazaki fragments synthesized. Although we did observe long-lived Pol  $\alpha^{AF647}$  binding events, they were exclusively associated with stalled forks (Figure S1C-D).

Second, we spiked 20 nM of Pol  $\delta^{AF647}$  into undepleted extract, which contains ~1000 nM of endogenous Pol  $\delta$ . We found that Pol  $\delta^{AF647}$  transiently bound to elongating replisomes and resided at forks for only ~15 seconds (Figure 1E-F, light blue). Unlike Pol  $\alpha$ , Pol  $\delta$  was not retained at stalled forks (Figure S1E).

Short Pol  $\alpha/\delta$  residence times may reflect concentration-dependent exchange as reported for polymerases in yeast<sup>18</sup>. Endogenous Pol  $\alpha$  (~700 nM) and Pol  $\delta$  (~1000 nM) concentrations in undepleted extract are comparable to Pol  $\alpha$  (360-630 nM) and Pol  $\delta$  (300-650 nM) concentrations reported in human cells<sup>25</sup>. To determine the lowest polymerase concentration that supports normal replication, we performed an ensemble replication assay and found that Pol  $\alpha/\delta$  concentrations above ~50 nM supported replication as well as undepleted extract (Figure S1F-I). Polymerase concentrations below 50 nM resulted in elevated pCHK1 levels relative to mock-depleted controls (Figure S1J-K).

To test whether polymerase exchange is concentration-dependent, we repeated the spike-in experiment using Pol  $\alpha$ -depleted extract supplemented with 5 nM of Pol  $\alpha^{AF647}$  and 45 nM of unlabeled Pol  $\alpha$  (50 nM in total – the lowest concentration that supports normal replication). Lowering the Pol  $\alpha$  concentration to 50 nM did not significantly change its residence time at the fork (Figure 1G, 1D, dark green).

We performed a similar experiment in Pol  $\delta$ -depleted extract supplemented with 5 nM of Pol  $\delta^{AF647}$  and 45 nM of unlabeled Pol  $\delta$  and found that Pol  $\delta^{AF647}$  bound to forks for only ~22 sec (Figure 1H, 1F, dark blue). Pol  $\delta$ 's residence time is different from that of Pol  $\alpha$ , suggesting that they do not form a stable complex as hypothesized previously<sup>26,27</sup>. Consistent with this, Pol  $\alpha$  and Pol  $\delta$  co-IP only weakly (Figure S1L).

Stretching the DNA template in our experiments may disfavor the formation of trombone-like loops<sup>15,16</sup>, and favors the separation of sister replisomes<sup>28</sup> (contrasting with reports that sister replisomes may remain physically coupled *in vivo*<sup>29</sup>). To address this, we measured the residence time of Pol  $\alpha/\delta$  on singly-tethered DNA molecules that are not under tension. These DNAs are rapidly chromatinized and compacted into a diffraction-limited spot (Figure 1B, dotted circle). We found that Pol  $\alpha/\delta$  reside on replicating single-tethered DNA for ~43 seconds (Figure S1M-O) and 18 seconds (Figure S1P-R) respectively, consistent with the *distributive* mechanism. These data indicate that neither Pol  $\alpha$  nor Pol  $\delta$  reside at the fork long enough to synthesize several consecutive Okazaki fragments, regardless of their total concentrations.

### Binding of a Single Copy of Inactive Pol $\alpha/\delta$ to the Fork Does Not Impair Lagging Strand Synthesis

The trombone model predicts that the incorporation of a single inactive Pol  $\alpha/\delta$  into the replisome should block lagging strand synthesis for several minutes. To test this prediction, we generated catalytically dead Pol  $\alpha$  (Pol  $\alpha^{Dead}$ ) by mutating the POLA1 subunit (D998S, D1000S) as described before<sup>18</sup> (Figure 2A). Notably, Pol  $\alpha^{Dead}$  retains its RNA primase activity<sup>18</sup>. We verified that Pol  $\alpha^{Dead}$  cannot rescue replication in a Pol  $\alpha$ -depleted extract (Figure 2B, S2A). Spiking 20 nM of fluorescently labeled Pol  $\alpha^{Dead}$  into undepleted extract revealed that it resides

at forks as long as wildtype Pol  $\alpha$  (Figure 2C-D). Importantly, the recruitment of Pol  $\alpha^{\text{Dead}}$  to the replisome did not impair lagging strand synthesis as monitored by growing FEN1<sup>mKikGR</sup> tracts (Figure 2C). We verified that the fluorescent tag does not affect Pol  $\alpha$ 's activity (Figure S2B-C).

Similarly, we generated catalytically dead Pol  $\delta$  (Pol  $\delta^{\text{Dead}}$ ) by mutating POLD1 (D606A, D761A) as previously described<sup>9</sup> (Figure 2E). We confirmed that Pol  $\delta^{\text{Dead}}$  cannot rescue replication in Pol  $\delta$ -depleted extract (Figure 2F, S2D). Spiking 20 nM of fluorescently-labeled Pol  $\delta^{\text{Dead}}$  into undepleted extract revealed that it binds forks (Figure 2G), but resides on DNA much longer than wildtype Pol  $\delta$  (Figure 2H). This is consistent with previous reports that human Pol  $\delta^{\text{Dead}}$  dissociates from its template slowly<sup>9,30</sup>. We verified that the fluorescent tag does not affect Pol  $\delta$ 's activity (Figure S2E-F). Strikingly, although Pol  $\delta^{\text{Dead}}$  initially bound to the edge of the FEN1<sup>mKikGR</sup> bubble, Pol  $\delta^{\text{Dead}}$  did not travel with the advancing fork (Figure 2G). This suggests that Pol  $\delta$  is deposited behind the fork (Figure S2G) and corroborates the idea that Pol  $\delta$  is not stably tethered to the replisome.

These findings indicate that the replisome can tolerate the recruitment of a single inactive Pol  $\alpha/\delta$  without impairing lagging strand synthesis.

### **Lagging Strand Synthesis Stops Shortly After Chasing with Extract Depleted of Pol $\alpha/\delta$**

The surprisingly short residence time of Pol  $\alpha/\delta$  on nascent DNA (Figure 1D, 1F) suggests that these polymerases rapidly dissociate from the fork and that new molecules of Pol  $\alpha/\delta$  are frequently recruited from solution to sustain lagging strand synthesis. If so, processive lagging strand synthesis should require an abundant pool of soluble Pol  $\alpha/\delta$ . To test this hypothesis, we performed a chase experiment where we initiated replication in undepleted extract, then flushed the flow-cell with mock-depleted, Pol  $\alpha$ -depleted, or Pol  $\delta$ -depleted extract (Figure 3A).

Chasing with mock-depleted extract did not affect the growth of FEN1<sup>mKikGR</sup> tracts, indicating that the chase did not impair lagging strand synthesis (Figure 3B-C). In contrast, chasing with Pol  $\alpha$ -depleted or Pol  $\delta$ -depleted extract abruptly halted FEN1<sup>mKikGR</sup> tract growth 1-2 min after starting the chase (Figure 3D-I). We verified that the chase removed >99% of endogenous Pol  $\alpha/\delta$  from the flow-cell (Figure S3A-B). These experiments indicate that Pol  $\alpha/\delta$  are not recycled at the fork and that processive lagging strand synthesis cannot occur in the absence of soluble polymerases. Importantly, this sudden halt in lagging strand synthesis is not caused by ATR/CHK1 activation, as there is no detectable pCHK1 signal in extract collected from the flow-cell (Figure S3C, lanes 10 and 14), due to the low DNA concentration in these reactions.

We observed similar results when chasing with Pol  $\alpha/\delta$ -depleted extract supplemented with catalytically-dead Pol  $\alpha/\delta$  (Figure S3D-G). These experiments show that neither Pol  $\alpha$  nor Pol  $\delta$  are stably tethered to the replisome, but that new copies of Pol  $\alpha/\delta$  must be recruited to the fork to support the synthesis of new Okazaki fragments.

### **Leading and Lagging Strand Synthesis Are Not Intrinsically Coupled**

Our finding that Pol  $\alpha/\delta$  are not stably affixed to the replisome, suggests that leading and lagging strand synthesis are not intrinsically coupled. To test this prediction, we performed single-molecule chase experiments where we monitored ssDNA accumulation by imaging Replication Protein A (RPA) tagged with mKikGR (RPA<sup>mKikGR</sup>) and CMG dynamics by visualizing GINS labeled with Lumidyne555 (GINS<sup>LD555</sup>).

First, we chased with mock-depleted extract supplemented with DMSO (Figure 4A). During this chase, CMG speed didn't change and there was little RPA<sup>mKikGR</sup> signal at the fork (Figure 4B) because there is little exposed ssDNA during unperturbed replication.

Second, we chased with mock-depleted extract containing 50  $\mu\text{M}$  of aphidicolin (a replicative polymerase inhibitor that blocks leading and lagging strand synthesis<sup>31-33</sup>). During this chase, CMG slowed down to ~40 nt/min and RPA<sup>mKikGR</sup> accumulated behind the helicase (Figure 4C). This is consistent with previous reports that CMG slows down ~10-fold when DNA synthesis is inhibited<sup>34</sup> and that fast replisome progression requires leading strand synthesis by Pol  $\epsilon$ <sup>3</sup>.

Third, we chased with extract containing 100  $\mu\text{M}$  of CD437 – a Pol  $\alpha$  inhibitor<sup>35</sup>. During this chase, a long tract of RPA<sup>mKikGR</sup> accumulated behind CMG (Figure 4D), consistent with RPA binding to the lagging strand template in the absence of Okazaki fragment synthesis. Notably, CMG did not slow-down during the CD437 chase, suggesting that blocking Okazaki fragment synthesis did not impair leading strand synthesis (Figure 4D, cartoon). We verified that nucleotide incorporation continued during the CD437 chase (Figure S4A-C). Because 100  $\mu\text{M}$  of CD437 ablates Pol  $\alpha$  activity (Figure S4D), any dNTP incorporation must come from leading strand synthesis.

Finally, we chased with Pol  $\alpha$ -depleted extract supplemented with 500 nM of Pol  $\alpha^{\text{Dead}}$ , which should rapidly block Okazaki fragment synthesis. Like the CD437 chase, chasing with Pol  $\alpha^{\text{Dead}}$  did not alter CMG speed, but resulted in massive accumulation of RPA<sup>mKikGR</sup> behind CMG (Figure 4E-F). These data indicate that the rate of leading strand synthesis is not intrinsically coupled to that of lagging strand synthesis.

Although lagging strand priming is suppressed during the Pol  $\alpha^{\text{Dead}}$  chase, we observed forks where the RPA tract was cleared in the opposite direction of CMG progression (Figure 4G). These events were rare (~1/100 molecules) and likely resulted from infrequent priming by trace amounts of wildtype Pol  $\alpha$  remaining after the chase. These RPA-clearing events are consistent with the idea that Pol  $\delta$  extends Okazaki fragments without being attached to the replisome.

## DISCUSSION

### Pol $\alpha$ and Pol $\delta$ Function *Distributively* During Lagging Strand Synthesis

Data presented here support a *distributive* mechanism of lagging strand synthesis in higher eukaryotes (Figure 4H). We find that Pol  $\alpha$  resides at the fork long enough for CMG to unwind 150-250 nt of template DNA (Figure 1D), indicating that new Pol  $\alpha$  molecules must be recruited to prime new Okazaki fragments. We find that Pol  $\delta$  is deposited behind the fork (Figure 2G), and that lagging strands are extended in the opposite direction of fork progression (Figure 4G), indicating that Pol  $\delta$  is not tethered to the replisome. Like Pol  $\alpha$ , new Pol  $\delta$  molecules must be recruited to extend new Okazaki fragments. As CMG continuously unwinds DNA, it exposes the lagging strand template and facilitates the recruitment of new Pol  $\alpha/\delta$  molecules for further rounds of *distributive* Okazaki fragment synthesis.

The *distributive* mechanism has several potential benefits. (i) It renders lagging strand replication robust to the binding of inactive Pol  $\alpha/\delta$  (Figure 2C, 2G). Since Pol  $\alpha/\delta$  contain Fe-S clusters that are essential for their enzymatic function<sup>36-38</sup>, endogenous or exogenous oxidative stress may inactivate some polymerases<sup>39</sup>. (ii) It does not require active mechanisms to slow-down leading strand synthesis if it outpaces lagging strand synthesis. (iii) It does not require the lagging strand template to form loops or any machinery to manage such loops. (iv) It may allow replisomes to pass through cohesin rings: CMG alone has a diameter of 10-15 nm<sup>40</sup>, so the persistent presence of Pol  $\alpha$  (12-15 nm)<sup>7</sup> and Pol  $\delta$  (7-10 nm)<sup>41</sup> may impede the ability of the replisome to pass through a cohesin ring (30-40 nm in diameter)<sup>42</sup>.

What causes Pol  $\alpha$  to dissociate from the replisome? In the absence of nucleotides, Pol  $\alpha$  forms a complex with CMG on forked DNA, and this interaction is mediated by multiple CMG-Pol  $\alpha$  contacts<sup>5</sup>. Mutating just one of these interfaces destabilizes the CMG-Pol  $\alpha$  interaction and reduces priming *in vitro*<sup>5</sup>. We speculate that the major conformational changes experienced by Pol  $\alpha$  during primer synthesis<sup>7,8</sup> disrupt Pol  $\alpha$ 's interaction with CMG, triggering its dissociation from the replisome. Future studies capturing CMG-Pol  $\alpha$  structures at different stages of primer synthesis are needed to test this prediction.

What causes Pol  $\delta$  to dissociate from the fork? It could be triggered by Pol  $\delta$ 's low intrinsic processivity, or by its collision with the previous Okazaki fragment<sup>11</sup>. We propose that Pol  $\delta$  is not tethered to CMG during Okazaki fragment elongation, similar to how it functions during homologous recombination<sup>43</sup> and break-induced replication<sup>44</sup>.

### Reconciling Our Observations with Previous Reports

A study tracking Pol  $\alpha/\delta$  dynamics in live yeast concluded that these polymerases bind chromatin long enough to synthesize many consecutive Okazaki fragments<sup>19</sup>. However, it remained unclear if the binding events measured in that study occurred at elongating forks. We find that Pol  $\alpha$  resides at active forks for only ~0.5 minutes compared to >2 minutes at stalled forks (Figure S1C-D). We surmise that some of the long-lived Pol  $\alpha$  binding events reported *in vivo*<sup>19</sup> occurred at stalled forks. Unlike Pol  $\alpha$ , we do not observe long-lived Pol  $\delta$  binding at stalled forks (Figure S1E). It is unclear if the long-lived binding events measured in yeast<sup>19</sup> reflect Pol  $\delta$  binding at forks or Pol  $\delta$  participating in DNA repair<sup>43-45</sup>. In human cells, Pol  $\delta$  colocalizes with PCNA for only ~4 seconds<sup>9</sup> (during which forks travel ~130 nucleotides in human cells), consistent with the *distributive* mechanism.

An *in vitro* reconstitution study found that yeast Pol  $\alpha/\delta$  travel with CMG long enough to synthesize >100 Okazaki fragments<sup>18</sup>. However, this study used polymerase concentrations 10-100x lower than in cells<sup>25,46</sup>, which cause ATR checkpoint activation in yeast<sup>47,48</sup> and in *Xenopus* nuclear extract (Figure S1J-K). We find that Pol  $\alpha/\delta$  function *distributively* across physiological concentrations (Figure 1D, 1F). Interestingly, the same study

found that a polymerase-dead but primase-proficient Pol  $\alpha$  (equivalent to Pol  $\alpha^{\text{Dead}}$  used here, Figure 2A) supported DNA replication *in vitro*<sup>18</sup>. This contrasts with our observation that Pol  $\alpha^{\text{Dead}}$  cannot support replication in extract (Figure 2B). We surmise that RNA primers synthesized by Pol  $\alpha^{\text{Dead}}$  are unstable in the nucleoplasm (and likely *in vivo*) due to RNases or other enzymes. Contrary to the previous report<sup>18</sup>, we conclude that Pol  $\alpha$ 's DNA polymerase activity is essential for lagging strand synthesis.

Previous studies found that decreasing Pol  $\alpha$ 's concentration *in vitro*<sup>3</sup> or *in vivo*<sup>47</sup> resulted in longer lagging strand fragments, which is difficult to reconcile with the trombone model. These reports are consistent with the *distributive* model: low Pol  $\alpha$  concentrations cause less frequent priming, resulting in longer Okazaki fragments.

It is possible that discrepancies between our findings and those from yeast studies<sup>18,19</sup> reflect real differences in lagging strand synthesis mechanisms in fungi versus metazoa. For example, Pol  $\delta$ 's interaction with PCNA is more stable in yeast than in metazoa<sup>49,50</sup>, and yeast Pol  $\delta$ -PCNA is more processive than its human counterpart<sup>11,51,52</sup>. There are also structural differences in the Pol  $\alpha$ -CMG interface between yeast and metazoa<sup>5</sup>. Finally, a recent study reported that Okazaki fragment maturation (which involves the coordinated action of Pol  $\delta$  and FEN1) is much slower in metazoa than in yeast<sup>53</sup>, consistent with our findings that Pol  $\delta$  does not travel with the fork, but remains behind CMG, where it can participate in lagging strand maturation.

### Comparing DNA Polymerase Dynamics in Eukaryotes versus Prokaryotes

Classic *in vitro* experiments demonstrated that prokaryotic DNA polymerases can be recycled for multiple Okazaki fragments under dilute conditions, when the recruitment of soluble polymerases is limiting<sup>16,54,55</sup>. However, high processivity under dilute conditions contrasts with lower processivity (i.e. more frequent polymerase exchange) at high concentrations of soluble polymerases<sup>56-61</sup>. To rationalize these discrepancies, a biophysical model was proposed to explain how a protein remains stably associated with a complex at low concentrations, yet exchanges rapidly with its counterparts in solution at high concentrations<sup>62</sup>. Such concentration-dependent exchange was measured via single-molecule imaging of reconstituted bacterial<sup>61</sup> and yeast<sup>18</sup> replisomes. However, even when polymerases exchange most frequently, individual bacterial DNA polymerases<sup>60,61</sup> or individual yeast Pol  $\alpha/\delta$ <sup>18</sup> are reused for several consecutive Okazaki fragments.

By contrast, when we recapitulated conditions that should favor polymerase recycling (chasing with polymerase-depleted extract), we did not observe processive Okazaki fragment synthesis (Figure 3D, G). This suggests that unlike prokaryotic and fungal systems, metazoan lagging strand replication is intrinsically non-processive. The need for metazoa to constantly recruit new Pol  $\alpha/\delta$  molecules during replication may explain why high concentrations of Pol  $\alpha/\delta$  are present in human cells (~300-600 nM)<sup>25</sup>. For example, there are ~200,000 copies of Pol  $\alpha/\delta$  in each human cell<sup>25</sup>, compared to only a few thousand active replication forks at any time<sup>63,64</sup>. This vast excess of lagging strand DNA polymerases is likely needed to ensure that Pol  $\alpha/\delta$  are rapidly recruited during Okazaki fragment synthesis.

### Leading Strand Synthesis and Lagging Strand Synthesis Are Not Intrinsically Coupled

It was previously thought that leading and lagging strand synthesis may be intrinsically coordinated to prevent ssDNA accumulation on the lagging strand template<sup>65-68</sup>. We find that there is no such intrinsic coupling in metazoa (Figure 4F). Instead, our data support a model where the lagging strand machinery is rapidly recruited to forks ensuring that Okazaki fragment synthesis keeps up with the rate of leading strand synthesis. Our findings are consistent with *in vitro* reconstitution studies reporting that fast leading strand synthesis can proceed in the absence of lagging strand synthesis for yeast<sup>3</sup> and human<sup>4</sup> replisomes.

There is conflicting data regarding the coupling of leading and lagging strand synthesis in human cells: one study found that treating cells with low doses of the Pol  $\alpha$  inhibitor CD437 does not impair leading strand synthesis<sup>69</sup>, while another study found that higher doses of CD437 activate ATR and slow-down forks, in part via fork reversal<sup>70</sup>. Given that ATR activation is stimulated by primer-template junctions generated by Pol  $\alpha$ <sup>13</sup>, it is unclear if these studies utilized CD437 concentrations sufficient to ablate Pol  $\alpha$  activity. Consistent with this idea, another study in human cells found that very high doses of Pol  $\alpha$  inhibitor suppress ATR activation<sup>71</sup>. Since there is no checkpoint signaling in our single-molecule experiments (Figure S3C), we demonstrate that metazoan leading and lagging strand synthesis are not intrinsically coupled. Such uncoordinated replication was also observed in prokaryotes<sup>72-74</sup> suggesting a convergent evolution of this mechanism.

## Limitations of the Study

Immunodepleting Pol  $\delta$  co-depleted ~90% of Topoisomerase II  $\alpha$  (Topo II  $\alpha$ ), causing a defect in plasmid decatenation (Figure S4E-F) that was fully rescued by 200 nM of recombinant Topo II  $\alpha$  (Figure S4E-G). To mitigate this, we supplemented Pol  $\delta$ -depleted extract with 200 nM Topo II  $\alpha$  in all ensemble experiments.

Pol  $\alpha$ 's residence time at active forks is consistent with one Pol  $\alpha$  molecule priming one Okazaki fragment. We cannot exclude the possibility that occasionally, one Pol  $\alpha$  molecule may prime two consecutive Okazaki fragments, as we cannot directly measure individual lagging strand fragments in our single-molecule assay. We cannot rule out that several Pol  $\delta$  molecules are needed to extend each Okazaki fragment, as proposed previously<sup>9</sup>.

Except for chase experiments, there is no extract flow during single-molecule experiments. To minimize shear forces, extract flow rate was kept very low (10  $\mu$ L/min) during chases, resulting in no distortion of double-tethered DNAs (Figure 3B).

Forks are slower in our experiments (~300-500 nt/min) than in human cells (~1,000-2,000 nt/min) because cells are cultured at 37°C, while our experiments were performed at 24°C (*Xenopus* is a cold-adapted organism and extract experiments fail above ~26°C).

A small fraction (5-15%) of Pol  $\alpha/\delta$  binding events occur behind forks (Figure S4H-I) – they may represent polymerases binding to ssDNA gaps far behind CMG, or non-specific binding to the coverslip (Figure S4J-K).

## RESOURCE AVAILABILITY

### Lead Contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Gheorghe Chistol (chistol@stanford.edu).

### Materials Availability

Unique reagents generated and described during this study are available upon reasonable request. Some may require a completed materials transfer agreement and compensation for shipping costs.

### Data and Code Availability

- Raw data generated for this study has been deposited to the Stanford Digital Repository, and is publicly available at <https://doi.org/10.25740/sq803bx0564>.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## ACKNOWLEDGEMENTS

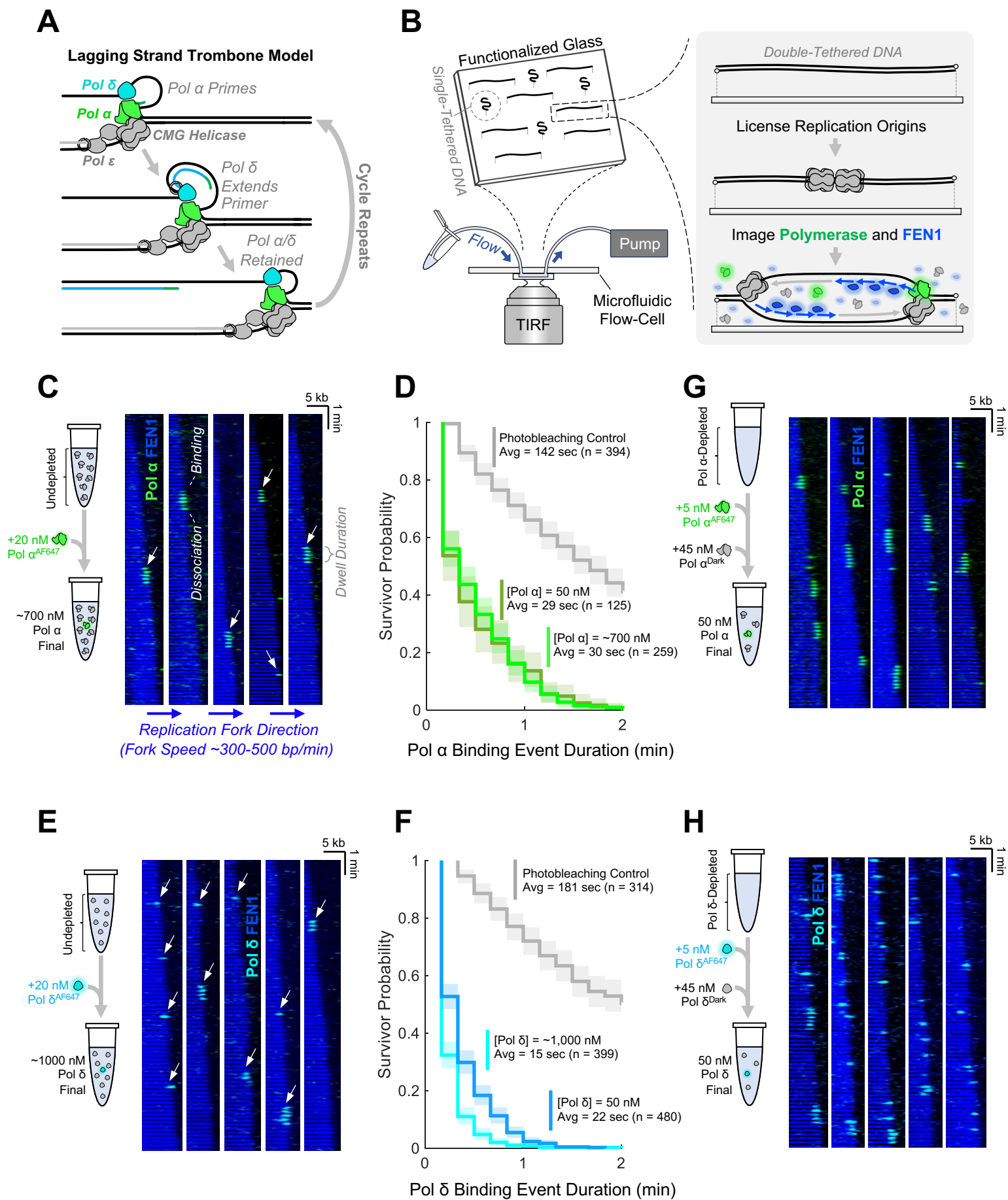
We thank Karlene Cimprich, Joanna Wysocka, Johannes Walter, David Pellman, Riki Terui, Gongshi Bai, Ulrike Kuehbach, NaYoung Cheon, Larissa Sambel, Zhitong Feng, and Scott Berger for critiques of the manuscript. We thank Riki Terui for cloning *Xenopus* RPA<sup>mKikGR</sup>. We thank Johannes Walter for the gift of an initial batch of anti-POLA1 serum. We thank James Dewar for the gift of affinity-purified anti-TOP2A antibodies. We thank Tatsuro Takahashi for the gift of sera against subunits of the FACT complex. We thank Tomasz Swigut for the bioinformatic search of POLD4 in *Xenopus*. L.D.L. was supported by NIH T32GM007276. G.C. is supported by NSF CAREER (2144481), NIGMSR35 (GM147060), and an American Cancer Society seed grant (228425). J.P. is supported by an American Cancer Society award, PF-26-1595977-01-PF MBBW25, Grant DOI #: [https://doi.org/10.53354/ACS.PF-26-1595977-01-PFMBB-W25.pc.gr.246507].

## AUTHOR CONTRIBUTIONS

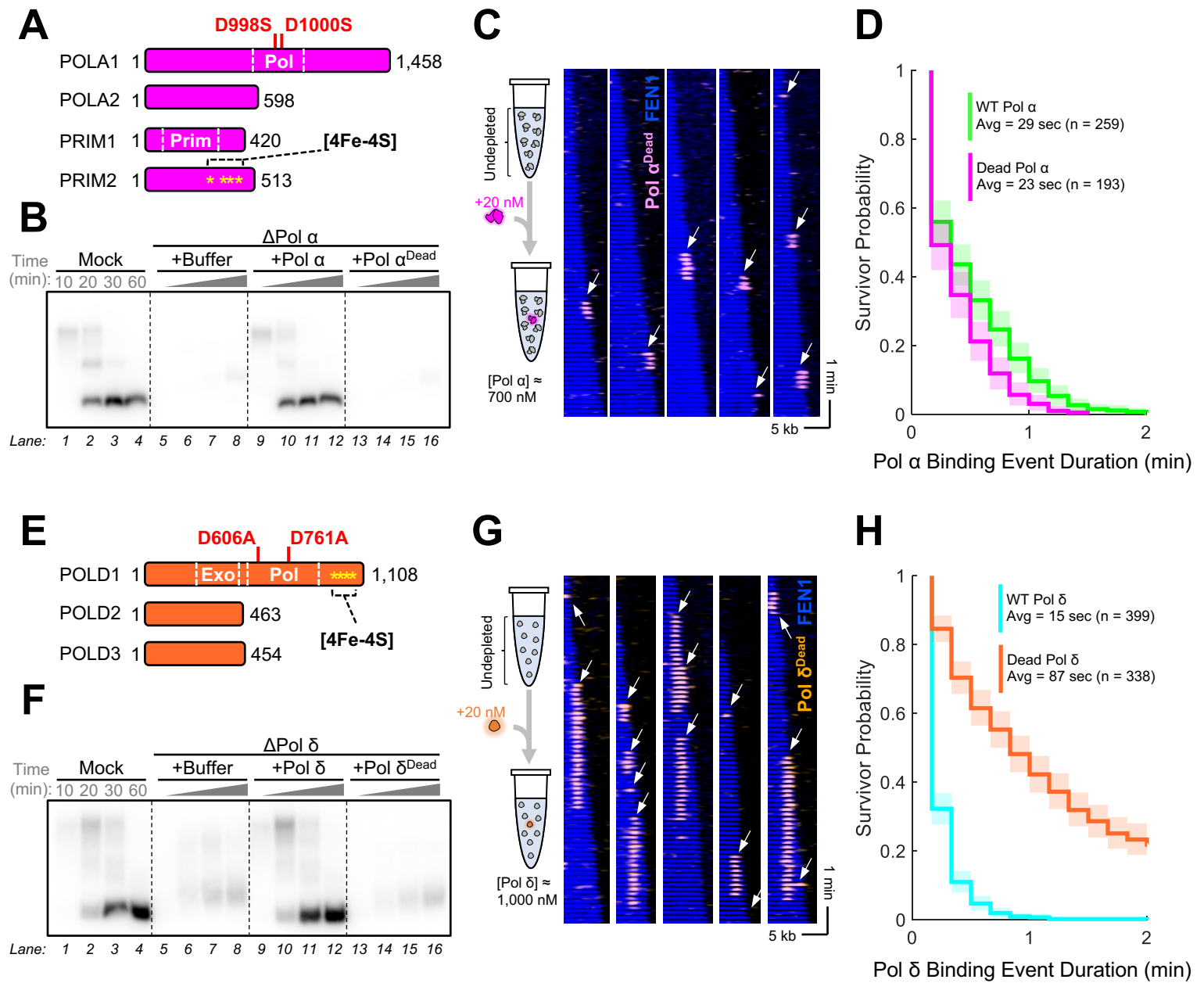
L.D.L. and G.C. conceived the project; L.D.L. carried out all experiments except for those in Figure S4B-C, which were carried out by J.P.; J.P. expressed/purified *Xenopus* RPA<sup>mKikGR</sup> and affinity-purified anti-POLA1 antibodies; G.C. and L.D.L. wrote MATLAB code for data analysis; L.D.L. analyzed all data with input from G.C. and J.P.; L.D.L. and G.C. wrote the manuscript, with input from J.P.

## DECLARATION OF INTERESTS

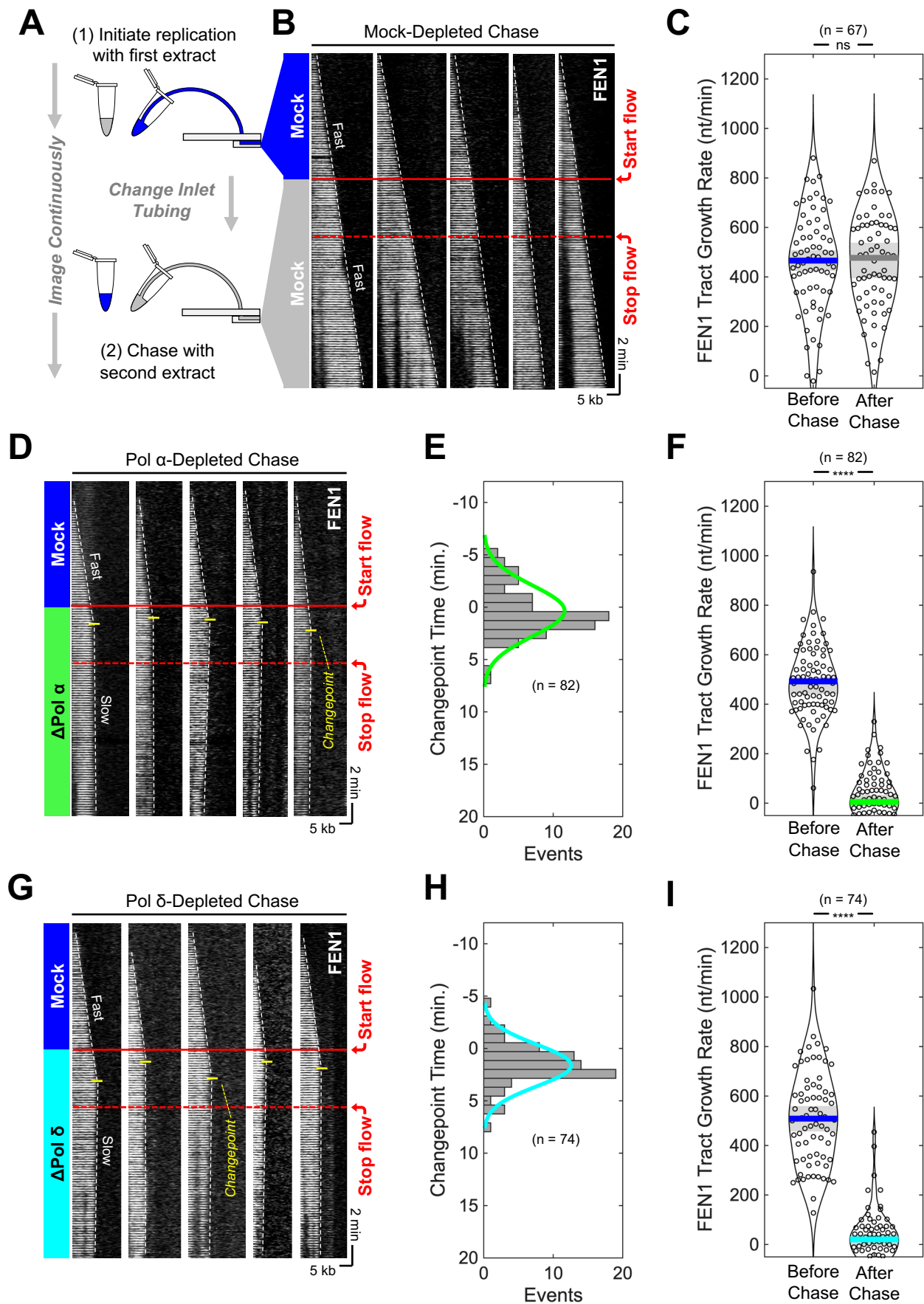
# Figure 1



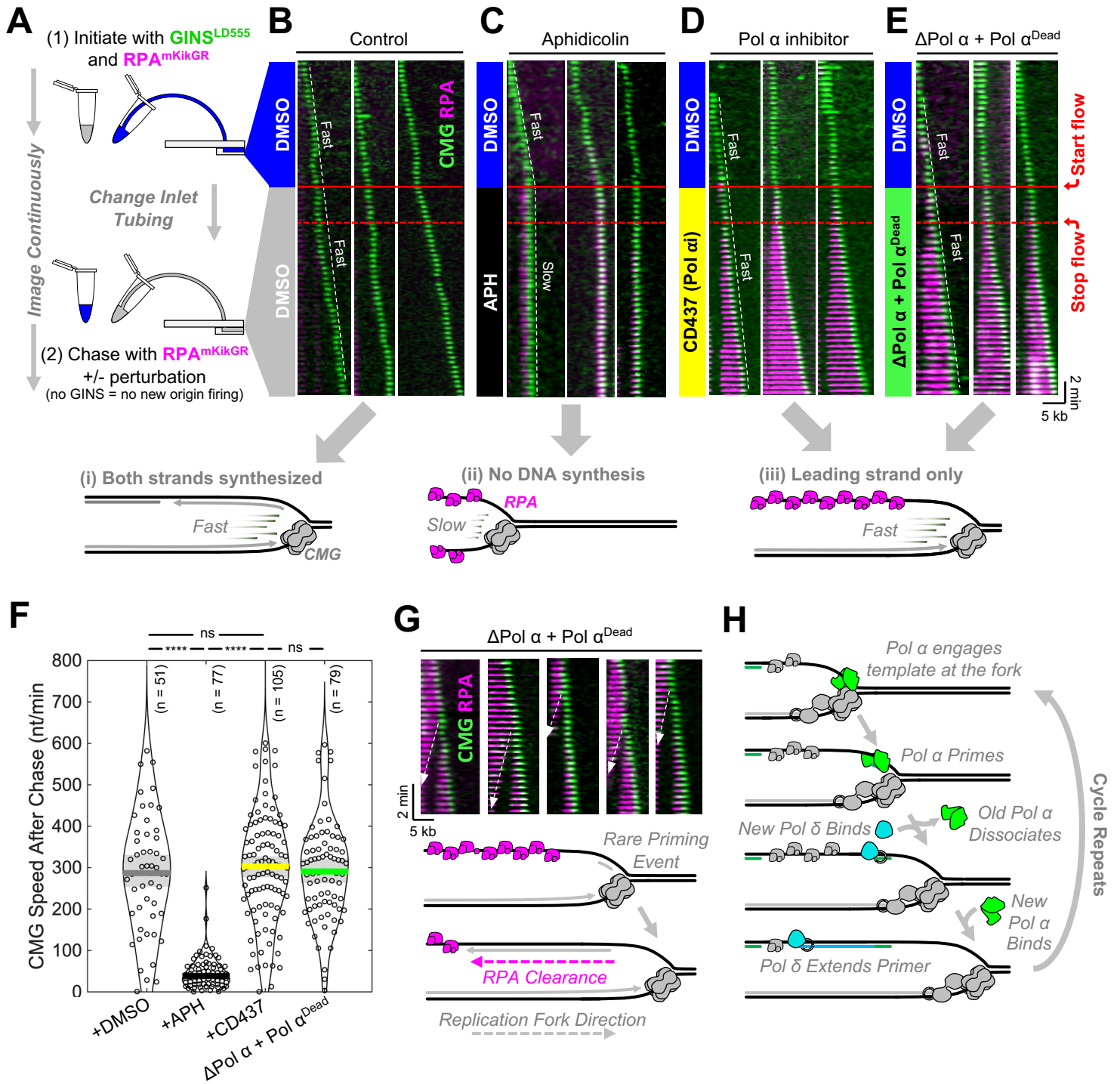
# Figure 2



# Figure 3



# Figure 4



Authors declare no conflicts of interest.

## MAIN FIGURE TITLES AND LEGENDS

### Figure 1: Pol $\alpha/\delta$ Are Not Stably Tethered to the Replisome

(A) Cartoon of the trombone model. (B) Single-molecule experiment workflow. (C) Kymograms of Pol  $\alpha^{AF647}$  (green) and FEN1<sup>mKikGR</sup> (blue) where 20 nM of Pol  $\alpha^{AF647}$  was spiked into undepleted extract containing ~700 nM of endogenous Pol  $\alpha$ . (D) Survivor probability curves for Pol  $\alpha^{AF647}$  binding event duration. AF647 photobleaching times (gray) are much longer than Pol  $\alpha^{AF647}$  residence time at forks (dark/light green). (E) Kymograms of Pol  $\delta^{AF647}$  (cyan) and FEN1<sup>mKikGR</sup> (blue) where 20 nM of Pol  $\delta^{AF647}$  was spiked in to undepleted extract containing ~1,000 nM of endogenous Pol  $\delta$ . (F) Survivor probability curves for Pol  $\delta^{AF647}$  binding event duration. Pol  $\delta^{AF647}$  residence time at forks (dark/light blue) is much shorter than the photobleaching control (gray). (G) Kymograms of Pol  $\alpha^{AF647}$  (green) and FEN1<sup>mKikGR</sup> (blue) where 5 nM Pol  $\alpha^{AF647}$  was spiked into Pol  $\alpha$ -depleted extract supplemented with 45 nM unlabeled Pol  $\alpha$ . (H) Kymograms of Pol  $\delta^{AF647}$  (green) and FEN1<sup>mKikGR</sup> (blue) where 5 nM Pol  $\delta^{AF647}$  was spiked into Pol  $\delta$ -depleted extract supplemented with 45 nM unlabeled Pol  $\delta$ . In (D) and (F), boxes depict the 95% Confidence Interval (CI) estimated via bootstrapping; 'n' denotes the number of events. See also Figure S1.

### Figure 2: A Single Copy of Catalytically-Dead Pol $\alpha/\delta$ Does Not Impair Okazaki Fragment Synthesis

(A) Diagram of catalytically-dead *Xenopus* Pol  $\alpha$ . (B) Plasmid replication assay: Pol  $\alpha$ -depleted extract was supplemented with 100 nM of recombinant Pol  $\alpha^{WT}$  or Pol  $\alpha^{Dead}$ . (C) Kymograms where 20 nM of fluorescent Pol  $\alpha^{Dead}$  (magenta) was spiked into undepleted extract containing ~700 nM of endogenous Pol  $\alpha$ . (D) Survivor probability curves of binding event duration for Pol  $\alpha^{Dead}$  (magenta) versus Pol  $\alpha^{WT}$  (green). (E) Diagram of catalytically-dead *Xenopus* Pol  $\delta$ . (F) Plasmid replication assay: Pol  $\delta$ -depleted extract was supplemented with 100 nM of recombinant Pol  $\delta^{WT}$  or Pol  $\delta^{Dead}$ . (G) Kymograms where 20 nM of fluorescent Pol  $\delta^{Dead}$  (orange) was spiked into undepleted extract containing ~1,000 nM of endogenous Pol  $\delta$ . (H) Survivor probability curves for binding event duration: Pol  $\delta^{Dead}$  (orange) versus Pol  $\delta^{WT}$  (cyan). In (D) and (H), boxes depict the 95% CI; 'n' denotes the number of events. See also Figure S2.

### Figure 3: Chasing with Pol $\alpha/\delta$ -Depleted Extract Rapidly Blocks Okazaki Fragment Synthesis

(A) Diagram of single-molecule chase experiments. (B) Kymograms of FEN1<sup>mKikGR</sup> (gray) in a chase experiment with mock-depleted extract. Red lines mark the start/stop of chase flow. (C) The growth rate of FEN1<sup>mKikGR</sup> tracts before/after the mock chase. (D) Kymograms from a chase with Pol  $\alpha$ -depleted extract. Dashed line marks the edge of FEN1 tracts fit by two lines with a changepoint (yellow). (E) Changepoint time for the chase with Pol  $\alpha$ -depleted extract (zero denotes the chase start). (F) The growth rate of the FEN1<sup>mKikGR</sup> tracts before/after chasing with Pol  $\alpha$ -depleted extract. (G) Kymograms from a chase with Pol  $\delta$ -depleted extract. (H) Changepoint time for the chase with Pol  $\delta$ -depleted extract. (I) The growth rate of FEN1<sup>mKikGR</sup> tracts before/after chasing with Pol  $\delta$ -depleted extract. In (C), (E), (F), (H), and (I) 'n' denotes the number of forks. In (C), (F), and (I), 'ns' denotes  $p > 0.05$ , '\*\*\*\*' denotes  $p < 0.0001$  (computed via Kolmogorov-Smirnov test); boxes represent 95% CI; lines represent the median. In (E) and (H) green/cyan curves represent Gaussian fits. See also Figure S3.

### Figure 4: Leading and Lagging Strand Synthesis are Not Intrinsically Coupled

(A) Single-molecule chase experiment workflow. (B) Kymograms of CMG (green) and RPA<sup>mKikGR</sup> (magenta) where the chase extract contains DMSO. CMG was labeled with GINS<sup>LD555</sup>. (C) Kymograms where the chase extract contains 50  $\mu$ M aphidicolin. (D) Kymograms where the chase extract contains 100  $\mu$ M CD437. (E) Kymograms where the chase contains Pol  $\alpha$ -depleted extract with 500 nM Pol  $\alpha^{Dead}$ . (F) CMG speeds measured after the chase. 'ns' denotes  $p > 0.05$ , '\*\*\*\*' denotes  $p < 0.0001$  (computed via Kolmogorov-Smirnov test). Horizontal lines and boxes represent the median and 95% CI. (G) Kymograms of CMG (green) and RPA<sup>mKikGR</sup> (magenta) from experiment in (E) depicting rare events where RPA is cleared away from CMG (cartoon illustrates how RPA is cleared). (H) Cartoon of proposed *distributive* mechanism of lagging strand synthesis. See also Figure S4.

## MAIN TABLES AND LEGENDS

None.

## STAR METHODS TEXT

### Key Resources Table

See Excel Spreadsheet

## Experimental Model and Subject Details

### Xenopus Laevis

Eggs from adult, female *Xenopus laevis* frogs (NASCO Healthcare, Cat# LM00531) were used to prepare egg extracts and the testes of adult, male *Xenopus laevis* frogs (NASCO Healthcare, Cat# LM00715MX) were used to prepare sperm chromatin. All animals were healthy, not subjected to previous procedures, and no animal husbandry was performed. Frogs were housed at the Boswell Small Amphibians Research Facility at Stanford School of Medicine in compliance with Institute Animal Care and Use Committee (IACUC) regulations and all experiments involving frogs were approved by the Stanford University APLAC (Administrative Panel on Laboratory Animal Care).

### Insect Cell Lines

Sf9 cells (Expression Systems, Cat# 94-001S) and Tni cells (Expression Systems, Cat# 94-002S) were cultured in ESF 921 insect cell culture medium (Expression Systems, Cat# 96-001-01) at 27°C for baculovirus production and protein overexpression.

### Bacterial Cell Lines Used

DH10EMBaY (Geneva Biotech, Cat# DH10EMBaY), DH5 $\alpha$  (New England Biolabs (NEB), Cat# C2987H), and Rosetta (DE3) pLysS (Novagen, Cat# 70956-4) bacteria were cultured in LB broth (Thermo Fisher Scientific, Cat# BP9723) at 37°C for plasmid production and protein overexpression.

## Methods Details

### Preparation of DNA Substrates for Single-Molecule Studies

All single molecule experiments used doubly tethered  $\lambda$  phage DNA. The 48.5 kb DNA substrate as purchased from NEB (#N3011S) has 12-nt 5' ssDNA overhangs on both ends. We used the Klenow Fragment (3' to 5' exo-) polymerase (NEB, Cat# M0212S) to fill in the overhangs with biotin-dCTP (Thermo Fisher Scientific, Cat# 19518018) and biotin-dGTP (PerkinElmer, Cat# NEL541001EA). The product is separated with electrophoresis and electro-eluted from the gel before use in tethering. A step-by-step detailed protocol for preparing biotinylated  $\lambda$  DNA is available in<sup>75</sup>.

### Xenopus Egg Extract Preparation

*Xenopus laevis* egg extracts were prepared following published protocols<sup>34,76</sup>. Frogs were primed with 50 IU of human chorionic gonadotropin (CHORULON) (Merck, Cat# 133754) 5-7 days before extract preparation. To induce ovulation, 500 IU of CHORULON was injected 20-22 hr before extract preparation. High-speed supernatant (HSS) was prepared from 5-6 adult female frogs, and nucleoplasmic extract (NPE) was prepared from 15-20 frogs. Sperm chromatin was purified from adult male frog testes and used for NPE preparation.

### Ensemble Replication Assay

To validate antibodies and recombinant proteins, ensemble DNA replication experiments were performed following a detailed protocol described in a recent methods article<sup>75</sup>. Since all proteins of interest (POIs) in this study are dispensable for Mcm2-7 double-hexamer loading, all licensing reactions were performed with HSS depleted of POI.

### Protein Co-Immunoprecipitation Assay

To test if Pol  $\alpha$  and Pol  $\delta$  form a complex in solution, 100 nM of each polymerase was mixed together in a 20  $\mu$ L reaction made up of ELB-Sucrose, 0.05% NP-40S, and 0.1 mg/mL BSA. The mixture was incubated for 1 hour

at room-temperature with gentle rotation. 10 µg of affinity-purified anti-POLD1 antibody was incubated with 60 µg Dynabeads Protein A (Invitrogen, Cat# 10002D) for 1 hour at room-temperature with gentle rotation. Beads were washed twice with ELB-Sucrose + 0.05% NP-40S and then incubated with the polymerase mixture and incubated for 1 hour at room-temperature with gentle rotation. Beads were washed three times with ice-cold ELB-Sucrose + 0.05% NP-40S. Bound proteins were eluted by boiling beads in 15 µL 1x Laemmli buffer (50mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, 2.5% β-mercaptoethanol).

### Western Blotting and Coomassie Staining

All samples were boiled in 1x Laemmli buffer and run on 4-15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad #4561086) using Tris-Glycine-SDS Running Buffer (Boston BioProducts #BP-150). EZ-Run Prestained Rec Protein Ladder (Fisher Scientific #BP36031) or Precision Plus Protein Dual Color Standard (Bio-Rad #1610374EDU) was run alongside samples to infer protein band sizes. For Coomassie staining, protein gels were incubated with Optiblot Blue InstantBlue Coomassie Protein Stain (Abcam #ab119211) for at least 1 hour at room temperature with gentle shaking. For immunoblotting, proteins were transferred from the gel onto a PVDF membrane (VWR #10061-492) in transfer buffer (Fisher Scientific #NC9297917). Membranes were blocked in 1x TBST (1x Tris Buffered Saline (TBS) (25 mM Tris pH 7.4, 150 mM NaCl), 0.1% Tween 20) containing 5% (w/v) BSA for 1 hour at room temperature with gentle shaking. Following a wash in 1xTBST, the membrane was incubated with primary antibodies diluted in 1x TBST containing 5% (w/v) BSA overnight at 4°C with gentle shaking. Membranes were washed three times with 1x TBST and incubated with secondary antibody diluted in 1x TBST + 5% BSA for 1 hour at room temperature with gentle shaking. Membranes were washed three times with 1x TBST, developed using SuperSignal West Pico PLUS Chemiluminescent Substrate (Fisher Scientific, Cat# PI34577), and imaged using Azure c600 (Azure Biosystems).

### Cloning and Plasmids

To prepare an antigen consisting of *Xenopus laevis* POLA1 amino acids 1 – 340, the corresponding DNA sequence was PCR amplified out of *X. laevis* egg cDNA. The resulting PCR product was assembled into pET28b(+) using the NEBuilder HiFi Mix (NEB, Cat# E2621S), yielding plasmid pJP006.

To prepare an antigen consisting of *X. laevis* POLD1 amino acids 1 – 89, the corresponding DNA sequence was PCR amplified out of *X. laevis* egg cDNA. The resulting PCR product was assembled into pET28b(+) using the NEBuilder HiFi Mix (NEB, Cat# E2621S), yielding plasmid pLDL048.

To prepare full-length *X. laevis* Pol α individual subunits were first cloned out of cDNA. The *polα1* gene was PCR amplified out of cDNA and assembled into BamHI/HindIII linearized pLib, yielding plasmid pLDL152. The *polα2* gene was PCR amplified out of cDNA and assembled into BamHI/HindIII linearized pLib, yielding plasmid pLDL153. The *prim1* gene was PCR amplified out of cDNA using primers and assembled into BamHI/HindIII linearized pLib, yielding plasmid pLDL154. The *prim2* gene was PCR amplified out of cDNA and assembled into BamHI/HindIII linearized pLib, yielding plasmid pLDL155. To tag the N-terminus of POLA1 with a TwinStrep-3aaLinker-HRV3C-S6-10aaLinker cassette, the *polα1* gene was PCR amplified out of pLDL152 and the resulting PCR product was assembled with a geneblock (synthesized by IDT) into BamHI/HindIII linearized pLib yielding plasmid pLDL156. All four subunits were then cloned into a single plasmid using the biGBac multigene construction strategy<sup>77</sup>. *twinstrep-3aalinker-hrv3c-s6-10aalinker-polα1* was PCR amplified out of pLDL156 using primers oLDL108/oLDL109, *polα2* was PCR amplified out of pLDL153 using primers oLDL110/oLDL111, *prim1* was PCR amplified out of pLDL154 using primers oLDL112/oLDL113, and *prim2* was PCR amplified out of pLDL155 using primers oLDL114/oLDL117. The four resulting PCR products were assembled into SmaI linearized pBiG1a, yielding the complete insect expression plasmid pLDL157.

To prepare a polymerase dead *X. laevis* Pol α, essential aspartic acids in the DNA polymerase active site of POLA1 were mutated to serine as previously described for the *Saccharomyces cerevisiae* ortholog<sup>18</sup>. D996 and D998 from *S. cerevisiae* Pol1 were mapped to D998 and D1000 in *X. laevis* POLA1.

To prepare full-length *X. laevis* Pol δ individual subunits were first cloned out of cDNA. The *pold1* gene was PCR amplified out of cDNA and assembled with a 3xflag-3aalinker-hrv3c-s6-10aalinker containing geneblock (synthesized by IDT) into pACEBac1, yielding plasmid pLDL148. The *pold2* gene was PCR amplified out of cDNA and assembled into pIDC, yielding plasmid pLDL079. The *pold3* gene was PCR

amplified out of cDNA using primers and assembled into pIDC, yielding plasmid pLDL080. All three subunits were then cloned into a single plasmid using the MultiBac multigene construction strategy<sup>78</sup>. The *pold3* gene was extracted from pLDL080 and inserted into linearized pLDL079, yielding pLDL081. The pLDL081 and pLDL148 vectors were fused by Cre-recombination, yielding pLDL149. The 3xFLAG tag was replaced by a TwinStrep tag via Gibson assembly, yielding the complete insect expression plasmid pLDL158.

To prepare a polymerase dead *X. laevis* Pol  $\delta$ , essential aspartic acids in the DNA polymerase active site of POLD1 were mutated to alanine as previously described for the *Homo sapiens* ortholog<sup>9</sup>. D602 and D757 from *H. sapiens* POLD1 were mapped to D606 and D761 in *X. laevis* POLD1.

To prepare full-length *X. laevis* Topo II  $\alpha$  the gene for *top2a* was cloned out of cDNA and inserted into a PCR linearized pLib backbone that already contained the DNA sequence for an upstream *twinstrep-HRV3C* cassette, yielding the insect expression plasmid pLDL189.

To prepare full-length *X. laevis* GINS with an S6 tag, a pGC124 containing *psf3-10aaLinker-sort-6xhis* and *sld5* was used as a starting point. A *10aalinker-s6-6xhis* cassette was added to the C-terminus of *psf3*, yielding the *psf3-10aaLinker-s6-6xhis* and *sld5* containing plasmid pLDL112. The pLDL112 (*psf3-10aaLinker-s6-6xhis/sld5*) and pGC033 (*psf1/psf2*) vectors were fused by Cre-recombination, yielding the complete GINS expression plasmid pLDL113.

## Antibody Preparation

Custom polyclonal antibodies were generated as previously described<sup>75</sup>. Briefly, the His6 tagged antigen sequence was cloned into pET28b(+) (see Cloning and Plasmids). The antigen was expressed in Rosetta (DE3) pLysS (Novagen, Cat# 70956-4) upon induction with 0.5-1.0 mM IPTG for 3 hrs at 37°C. Soluble antigens were purified in native buffer, whereas insoluble antigens were purified in denaturing buffer (see table and ‘Recombinant Protein Expression and Purification’ below). The Ni-NTA eluate (still containing 400 mM imidazole) was used to immunize rabbits. All antibodies used in this study were raised by Cocalico Biologicals.

All antibodies used in this study were affinity purified. 1 – 5 mg of purified antigen was cross-linked to 1 mL of AminoLink Plus Coupling Resin (Fisher Scientific, Cat# PI20501) according to the manufacturer’s protocol. 10 – 30 mL of serum was incubated with the affinity resin for 30 minutes at room temperature. The resin was washed twice with 10 mL of 1x PBS + 500 mM NaCl, and twice again with 10 mL of 1x PBS. Next, antibodies bound to the affinity resin were eluted 5-6 times with 1.0 mL of 200 mM glycine-HCl pH2.5. Each elution was collected into a tube containing 0.1 mL of 1M Tris-HCl pH 9.0, which immediately neutralized the glycine. Eluted fractions were pooled and dialyzed against 1 L of 1x TBS (50 mM Tris-HCl pH8.0, 150 mM NaCl) + 10% (w/v) sucrose. The IgG concentration was measured using a Nanodrop, normalized to 1 mg/mL via dilution or spin-concentration, aliquots were flash frozen in liquid nitrogen, and stored at -80°C.

| Target Protein<br>(Rabbit #) | Antigen                                   | Plasmid               | Antigen<br>Purification | Depletion<br>Condition<br>$\mu$ g IgG per<br>$\mu$ L extract | Primary Ab.<br>Conc.<br>for WB |
|------------------------------|-------------------------------------------|-----------------------|-------------------------|--------------------------------------------------------------|--------------------------------|
| Anti-xGINS<br>(SU289)        | Whole GINS complex<br>Psf2-10aa-sort-His6 | pGC127<br>(insect)    | Native                  | 1:1                                                          | 0.2 $\mu$ g/mL                 |
| Anti-xPOLA1<br>(SU375)       | (1-340 aa)-His6                           | pJP006<br>(bacteria)  | Native                  | 2:1                                                          | 0.2 $\mu$ g/mL                 |
| Anti-xPOLD1<br>(SU329)       | (1-89 aa)-His6                            | pLDL048<br>(bacteria) | Native                  | 1:1                                                          | 0.2 $\mu$ g/mL                 |

## Recombinant Protein Expression and Purification

FEN1<sup>mKikGR</sup>, RPA<sup>mKikGR</sup>, and SFP Synthase were purified following previously published protocols<sup>23,79,80</sup>.

Pol  $\alpha$  was purified as follows. A bacmid was generated by transforming DH10EMBacY cells with [pLDL157] pBiG\_TwinStrep-HRV3C-S6-Pol  $\alpha$ , and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit (Zymo Research, Cat# D4049). Baculovirus was amplified in three 72-hour stages (P0, P1, P2) in Sf9 cells. TwinStrep-HRV3C-S6-Pol  $\alpha$  was expressed in 1 L of Tni suspension culture by infection with 2 mL of P2

baculovirus for 48 hrs. Cells were harvested, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in 100 mL of lysis buffer (25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT – bubbled with argon gas to remove dissolved oxygen) supplemented with 1x Pierce Protease Inhibitor EDTA-free (ThermoFisher Scientific, Cat# A32965) and lysed by Dounce homogenization. Lysate was cleared by ultracentrifugation at 35,000 rpm for 45 min at 4°C in a Beckman Type 70 Ti rotor (Beckman Coulter). Cleared lysate was incubated with 1 mL of Strep-Tactin®XT 4®Flow resin (IBA Lifesciences, Cat# 2-5010-010) for 1 hr at 4°C. The resin was washed with 3x10 mL lysis buffer. The protein was eluted from the resin using 4 mL buffer N supplemented with 50 µg/mL HRV3C Protease for 2 hours at 4°C on a rotator, and flash frozen in liquid nitrogen. Eluent was thawed and, diluted with 2 mL of no-salt buffer (25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT) to reduce the NaCl concentration to 200 mM, and loaded on a Mono Q 5/50 GL column (Cytiva, Cat# 17516601). The protein was eluted in 20 CVs off the column with a linear gradient of 200 mM – 1000 mM NaCl and eluted around ~400 mM NaCl. Peak fractions were pooled, spin concentrated to ~0.5 mL using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024) applied to a Superdex 200 Increase 10/300 gel-filtration column (Cytiva, Cat# 28-9909-44) equilibrated in 25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT and 150 mM NaCl. Peak fractions were pooled, spin concentrated to ~6 µM using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024), and frozen in liquid nitrogen and stored at -80 °C. Approximately 100 µg total protein was obtained from 1 L of Tni cells. The Pol α<sup>Dead</sup> mutant was purified as described for the wild-type protein.

Pol δ was purified as follows. A bacmid was generated by transforming DH10EMBacY cells with [pLDL158] pBiG\_TwinStrep-HRV3C-S6-Pol δ, and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit (Zymo Research, Cat# D4049). Baculovirus was amplified in three 72-hour stages (P0, P1, P2) in Sf9 cells. We initially attempted to express Pol δ in Tni Cells, however the POLD3 subunit was not recovered (presumably due to proteolysis). Instead, TwinStrep-HRV3C-S6-Pol δ was expressed in 2 L of Sf9 suspension culture by infection with 4 mL of P2 baculovirus for 65 hrs. Cells were harvested, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in 200 mL of lysis buffer (25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT – bubbled with argon gas to remove dissolved oxygen) supplemented with 1x Pierce Protease Inhibitor EDTA-free (ThermoFisher Scientific, Cat# A32965) and lysed by Dounce homogenization. Lysate was cleared by ultracentrifugation at 35,000 rpm for 45 min at 4°C in a Beckman Type 70 Ti rotor (Beckman Coulter). Cleared lysate was incubated with 1 mL of Strep-Tactin®XT 4®Flow resin (IBA Lifesciences, Cat# 2-5010-010) for 1 hr at 4°C. The resin was washed with 3x10mL lysis buffer. The protein was eluted from the resin using 4 mL buffer N supplemented with 50 µg/mL HRV3C Protease for 2 hours at 4°C on a rotator, and flash frozen in liquid nitrogen. Eluent was thawed and, diluted with 2 mL of no-salt buffer (25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT) to reduce the NaCl concentration to 200 mM, and loaded on a Mono Q 5/50 GL column (Cytiva, Cat# 17516601). The protein was eluted in 20 CVs off the column with a linear gradient of 200 mM – 1000 mM NaCl and eluted around ~350 mM NaCl. Peak fractions were pooled, spin concentrated to ~0.5 mL using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024) applied to a Superdex 200 Increase 10/300 gel-filtration column (Cytiva, Cat# 28-9909-44) equilibrated in 25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT and 150 mM NaCl. Peak fractions were pooled, spin concentrated to ~4 µM using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024), and frozen in liquid nitrogen and stored at -80 °C. Approximately 50 µg total protein was obtained from 2 L of Sf9 cells. The Pol δ<sup>Dead</sup> mutant was purified as described for the wild-type protein.

Topo II α was purified as follows. A bacmid was generated by transforming DH10EMBacY cells with [pLDL189] pLib\_TwinStrep-HRV3C-Topo II α, and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit (Zymo Research, Cat# D4049). Baculovirus was amplified in three 72-hour stages (P0, P1, P2) in Sf9 cells. TwinStrep-HRV3C-Topo II α was expressed in 1 L of Sf9 suspension culture by infection with 2 mL of P2 baculovirus for 65 hrs. Cells were harvested, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in 100 mL of lysis buffer (25 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 10% glycerol, 1 mM DTT) supplemented with 1x Pierce Protease Inhibitor EDTA-free (ThermoFisher Scientific, Cat# A32965) and lysed by Dounce homogenization. Lysate was cleared by ultracentrifugation at 35,000 rpm for 45 min at 4°C in a Beckman Type 70 Ti rotor (Beckman Coulter). Cleared lysate was incubated with 1 mL of Strep-Tactin®XT 4®Flow resin (IBA Lifesciences, Cat# 2-5010-010) for 1 hr at 4°C. The resin was washed with 3x10mL lysis buffer. The protein was eluted from the resin using 4 mL lysis buffer supplemented

with 50 µg/mL HRV3C Protease for 2 hours at 4°C on a rotator, and flash frozen in liquid nitrogen. 2 mL of eluent was thawed and, diluted with 4 mL of no-salt buffer (25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT) to reduce the NaCl concentration to 100 mM, and loaded on a Mono S 5/50 GL column (Cytiva, Cat# 17516801). The protein was eluted in 20 CVs off the column with a linear gradient of 100 mM – 1000 mM NaCl and eluted around ~300 mM NaCl. Peak fractions were pooled, spin concentrated to ~0.5 mL using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024) applied to a Superdex 200 Increase 10/300 gel-filtration column (Cytiva, Cat# 28-9909-44) equilibrated in 25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT and 150 mM NaCl. Peak fractions were pooled, spin concentrated to ~4 µM using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024), and frozen in liquid nitrogen and stored at –80 °C. Approximately 300 µg total protein was obtained from 1 L of Sf9 cells.

GINS was purified as follows. A bacmid was generated by transforming DH10EMBaY cells with [pLDL113] pACEBac1\_GINS-S6-6xHis, and the resulting bacmid was purified with the ZR BAC DNA Miniprep Kit (Zymo Research, Cat# D4049). Baculovirus was amplified in three 72-hour stages (P0, P1, P2) in Sf9 cells. GINS-S6-6xHis was expressed in 1/2 L of Tni suspension culture by infection with 2 mL of P2 baculovirus for 48 hrs. Cells were harvested, pelleted by centrifugation, and flash frozen in liquid nitrogen. Cell pellets were thawed and resuspended in 100 mL of lysis buffer (25 mM HEPES-NaOH pH 7.5, 500 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM PMSF, 20 mM Imidazole) supplemented with 1x Pierce Protease Inhibitor EDTA-free (ThermoFisher Scientific, Cat# A32965) and lysed by Dounce homogenization. Lysate was cleared by ultracentrifugation at 35,000 rpm for 45 min at 4°C in a Beckman Type 70 Ti rotor (Beckman Coulter). Cleared lysate was incubated with 0.5 mL of Nickel-NTA resin (Qiagen 30230) for 1 hr at 4°C. The resin was washed with 3x10 mL lysis buffer. The protein was eluted from the resin using 5x0.5mL lysis buffer supplemented with 300 mM Imidazole, and flash frozen in liquid nitrogen. Eluent was thawed and, diluted with no-salt buffer (25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT) to reduce the NaCl concentration to 150 mM, and loaded on a Mono Q 5/50 GL column (Cytiva, Cat# 17516601). The protein was eluted in 20 CVs off the column with a linear gradient of 150 mM – 800 mM NaCl and eluted around ~300 mM NaCl. Peak fractions were pooled, spin concentrated to ~0.5 mL using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024) applied to a Superdex 200 Increase 10/300 gel-filtration column (Cytiva, Cat# 28-9909-44) equilibrated in 25 mM HEPES-NaOH pH 7.5, 10% glycerol, 1 mM DTT and 150 mM NaCl. Peak fractions were pooled, spin concentrated to ~3 µM using the Amicon Ultra-4-10K column (Merck Millipore, Cat# UFC801024), and frozen in liquid nitrogen and stored at –80 °C. Approximately 2 mg total protein was obtained from 1/2 L of Tni cells.

### Fluorescent Labeling Using SFP Synthase

For AF647 labeling, 1 mg of Alexa Fluor 647 C2 maleimide (Thermo Fisher Scientific, Cat # A20347) was reacted with 2 mg CoA-SH (Sigma-Aldrich, Cat # 234101-100MG) in 100 mM sodium phosphate pH 7.0 for 1 hour in the dark on a rotator at room temperature. The resulting product was separated from unreacted dye and unreacted CoA-SH by preparative HPLC using a reverse-phase Eclipse XDB-C18 column (PN 977250-102, 21.2 x 250 mm cartridge, 7 µm). The sample was run with a gradient of 0–50% (vol/vol) acetonitrile in 0.1% (vol/vol) TFA/water over 30 min at 10 mL/min. The AF647-CoA product eluted around ~36% acetonitrile. Peak fractions were pooled, lyophilized, and resuspended to a final concentration of 1 mM in DMSO. For LD555 and LD655 labeling, LD555-CoA and LD655-CoA was purchased from Lumidyne Technologies.

Site-specific fluorescent labeling of the S6 tag was performed in between the chromatography steps described above. Immediately following ion-exchange chromatography the molar concentration of the POI was estimated by the integrated intensity of the Abs280 reading on the FPLC chromatogram using a theoretical extinction coefficient. The POI was spin concentrated to ~0.5 mL and supplemented with 10 mM MgCl<sub>2</sub>, a 2-fold molar excess of SFP synthase and a 10-fold molar excess of CoA conjugated dye. Reactions were incubated for 1 hour in the dark on a rotator at room temperature, then applied to a Superdex 200 Increase 10/300 gel-filtration column to separate unreacted fluorophore and SFP from the POI. The peak fractions were pooled and concentrated. Protein labeling efficiency was estimated based on absorbance measurements at 280 nm and the absorption peak for the respective dye. The biochemical activity of the labeled protein was tested following a published detailed protocol<sup>75</sup>.

### Ensemble Plasmid Replication Assay

All plasmid replication assays were conducted following a detailed protocol<sup>75</sup> using 4 ng/μL pBlueScript as the template DNA. Samples were taken at specified timepoints to monitor  $\alpha^{32}\text{P}$ -dCTP incorporation or for western blotting.

### Single Molecule Replication Assay

All single molecule experiments were conducted following a detailed protocol<sup>75</sup>, except for the following modifications. In the  $\lambda$  DNA tethering step, 20 pM of streptavidin<sup>AF647</sup> (Thermo Fisher Scientific, Cat# S21374) was added to the streptavidin solution. Streptavidin<sup>AF647</sup> spots were used as a fiducial marker for focusing immediately before starting imaging. After  $\lambda$  DNA tethering, DNA was licensed in HSS for 10 minutes. In this study, Replication Mix (10 μL of NPE, 10 μL of HSS, 3 - 6 μL of ELB-Sucrose, 1 μL of 600 ng/μL pBlueScript carrier plasmid DNA, 1 μL of ATP regeneration Mix, 1 μL of 70 μM FEN1<sup>mKikGR</sup> or 1 μL of 11 μM RPA<sup>mKikGR</sup>, and 1- 3 μL of fluorescently labeled protein of interest = 30 μL total) was flowed into the microfluidic chamber at a rate of 10 μL/min. The reaction was then imaged continuously (10-30 sec/frame) without washing out the free fluorescently labeled protein. Thus, fluorescent protein (5-20 nM) was present in the replication reaction during the entire duration of the experiment unless indicated otherwise. Optimal replication efficiency was achieved when the single-molecule replication reaction contained equal volumes of NPE, HSS, and buffer (ref Yardimci 2012). The concentrations of proteins examined in this study were estimated using quantitative western blots (see table below).

| <i>Protein of Interest</i>     | <b>Concentration in NPE (nM)</b> | <b>Concentration in Replication Reaction (nM)</b> |
|--------------------------------|----------------------------------|---------------------------------------------------|
| <b>Pol <math>\alpha</math></b> | ~2,100 nM                        | ~700 nM                                           |
| <b>Pol <math>\delta</math></b> | ~3,000 nM                        | ~1,000 nM                                         |

For single molecule “chase” experiments the same procedure described above was repeated through the licensing step. Following licensing Replication Mix was flowed into the microfluidic chamber to initiate the reaction. After the flow completed, the inlet tubing was changed to a Chase Mix without starting flow. The Streptavidin<sup>AF647</sup> spots were used as a fiducial marker for focusing immediately before starting imaging. 15 minutes into the imaging acquisition the Chase Mix was flowed into the microfluidic chamber at 10 μL/min during continuous imaging.

When the replication reaction was chased with POI-depleted extract (Figure 3), samples from (1) the undepleted extract, (2) the depleted extract, and (3) the microfluidic flow-cell channel were taken for western blotting to validate the effectiveness of the chase in washing out the POI. To take the flow-cell sample, immediately after finishing the experiment, the inlet tubing was ripped out, and 1 μL was withdrawn from the flow-cell channel using a fine pipet tip. Initial attempts to chase with Pol  $\alpha/\delta$ -depleted extract resulted in substantial quantities of Pol  $\alpha/\delta$  from the initial Replication Mix persisting in the flow-cell (data not shown). Increasing the volume of the chase flow was necessary to wash out >99% of Pol  $\alpha/\delta$ . In Figure 3 the reactions were chased with 55 μL of Pol  $\alpha/\delta$ -depleted Chase Mix. We found that smaller 20 μL chase volumes were sufficient to block lagging strand synthesis when the Chase Mix was supplemented with catalytically dead polymerase as a competitor (Figure S3D-G). We note that it is critically important to measure the contents of the flow-cell in these types of experiments, as failure to effectively wash out a POI will lead to experimental artifacts.

Pol  $\alpha/\delta$ -depleted chase flow-cell samples from Figure 3 were monitored for CHK1 phosphorylation by Western blot (Figure S3C lanes 10 and 14). Flow cell samples were taken 30 minutes after the chase flow occurred. Using identical depletion conditions, we monitored CHK1 phosphorylation in ensemble plasmid replication assays in (1) mock-depleted, (2) mock-depleted supplemented with 5 μM aphidicolin, (3) Pol  $\alpha$ -depleted, and (4) Pol  $\delta$ -depleted extract (Figure S3C) and took samples for Western blotting at timepoints comparable to when the flow-cell sample was taken.

For chase experiments in Figure 4, replication was initiated in GINS-depleted extract supplemented with 20 nM GINS<sup>LD655</sup> and ~367 nM RPA<sup>mKikGR</sup>. The reactions were chased with GINS-depleted extract supplemented with ~367 nM RPA<sup>mKikGR</sup> and DMSO, 50 μM Aphidicolin, 100 μM CD437, or Pol  $\alpha$ -depleted extract supplemented with 500 nM Pol  $\alpha^{\text{Dead}}$ . GINS<sup>LD655</sup> was used during the 500 nM Pol  $\alpha^{\text{Dead}}$  chase to avoid spectral

overlap (Pol  $\alpha^{\text{Dead}}$  was labeled with Alexa Fluor 647). All reactions received a matched final concentration of 0.8% DMSO.

For chase experiments in Figure S4A-C GINS-depleted extract was supplemented with 20 nM GINS<sup>LD555</sup> and ~367 nM RPA<sup>mKikGR</sup>. Replication reactions were chased with GINS-depleted extract supplemented with DMSO or 100  $\mu\text{M}$  CD437. 16.7  $\mu\text{M}$  dUTP<sup>AZD647</sup> was included in the chase flow. During this, GINS<sup>LD555</sup> and RPA<sup>mKikGR</sup> were imaged continuously. After 30 minutes the channels were washed with 50  $\mu\text{L}$  DNA Buffer [10 mM Tris pH 8.0, 50 NaCl, 0.5 mM EDTA] flowed at 10  $\mu\text{L}/\text{min}$ . The post-imaged dUTP<sup>AZD647</sup> was aligned to the GINS<sup>LD555</sup>/RPA<sup>mKikGR</sup> movie using the Streptavidin<sup>AF647</sup> fiducial spots.

The following settings were used for imaging FEN1<sup>mKikGR</sup>: TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 10 s/frame, 8% 488 nm laser power ( $\sim 60 \mu\text{W}$  power out of the objective,  $\sim 0.6 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 100, EM Conversion Gain = 1, 10 MHz at 16-bit read-out speed.

The following settings were used for imaging both Pol  $\alpha^{\text{AF647}}$  and Pol  $\delta^{\text{AF647}}$ : TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 10 s/frame, 25% 640 nm laser power ( $\sim 380 \mu\text{W}$  power out of the objective,  $\sim 3.8 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 100, EM Conversion Gain = 1, 10 MHz at 16-bit read-out speed.

The following settings were used for imaging RPA<sup>mKikGR</sup>: TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 30 s/frame, 10% 488 nm laser power ( $\sim 68 \mu\text{W}$  power out of the objective,  $\sim 0.68 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 100, EM Conversion Gain = 1, 10 MHz at 16-bit read-out speed.

The following settings were used for imaging GINS<sup>LD555</sup>: TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 30 s/frame, 25% 561 nm laser power ( $\sim 500 \mu\text{W}$  power out of the objective,  $\sim 5.0 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 100, EM Conversion Gain = 1, 10 MHz at 16-bit read-out speed.

The following settings were used for imaging GINS<sup>LD655</sup>: TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 30 s/frame, 25% 640 nm laser power ( $\sim 380 \mu\text{W}$  power out of the objective,  $\sim 3.8 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 100, EM Conversion Gain = 1, 10 MHz at 16-bit read-out speed.

The following settings were used for dUTP<sup>AZD647</sup>: TIRF arm set to 9000 ( $\sim 67^\circ$ ), 500 ms exposure, 10 s/frame, 25% 640 nm laser power ( $\sim 380 \mu\text{W}$  power out of the objective,  $\sim 3.8 \text{ W}/\text{cm}^2$  power density), EM Gain Multiplier = 150, EM Conversion Gain = 3, 10 MHz at 16-bit read-out speed.

### Single-Molecule Data Analysis

Each replication initiation experiment imaged 6-8 fields of view (FOVs) over the course of 45-60 minutes with a resolution of 10-30 seconds/frame. Each FOV typically contained 50-200 DNA molecules stretched to  $\sim 80\%$  of their contour length. A suite of MATLAB scripts written in house were used to select and analyze data as described in our recent methods article<sup>75</sup>. Below we summarize the data analysis pipeline specific for the single molecule experiments analyzed in this study.

### Selecting DNA Molecules for Downstream Analysis

Regions of interest (ROIs) corresponding to individual DNA molecules were selected manually based on the previews of DNA stained with SYTOX Orange (these were acquired before the start of the experiment). Only well-separated DNA molecules were used in the analysis.

### Analyzing Polymerase Binding/Dissociation Events

To analyze the binding and dissociation of polymerases we employed an approach previously described in<sup>81</sup> for analyzing replication initiation factors. ROIs corresponding to polymerase binding events were selected from edges of FEN1 tracts, corresponding to the position of the replisome. For the analysis of fork-distal binding events (Figure S4H-I) ROIs corresponding to polymerase binding events were selected from FEN1 tracts behind the fork, corresponding to nascent DNA that the replisome had already moved past.

To analyze the binding and dissociation of polymerases on singly tethered molecules (Figure S1M-R), ROIs were also selected from the DNA Preview – singly tethered molecules appear as diffraction limited sources of SYTOX signal. Only singly-tethered molecules that replicated (displayed FEN1 signal) were analyzed. To minimize the number of “non-productive” binding events (Figure S1C-D) analyzed, we applied the following criteria: total FEN1 signal was integrated over time. This signal increases as the molecule replicates but eventually peaks and decreases presumably due to completion of DNA replication triggering FEN1 unloading. Polymerase binding events were therefore only selected from time points prior to the peak in FEN1 signal.

### Changepoint Analysis

To identify the time when lagging strand synthesis ceased following chases in Figure 3, the edge of the FEN1 tract was fit with the following bilinear model in MATLAB:

$$b(x) = \begin{cases} a_1x + b & x < x_0 \\ a_2x + (a_1 - a_2)x_0 & x \geq x_0 \end{cases}$$

where  $x_0$  is the changepoint time,  $a_1$  is the slope before the changepoint,  $a_2$  is the slope after the changepoint, and  $b$  is the y-intercept. To confirm the robustness of the changepoint analysis we compared the goodness of fit to a linear model that lacks a changepoint term. The following monlinear model was fit to the same FEN1 tract:

$$m(x) = a_1x + b$$

For each model we approximated the Bayesian Information Criterion (BIC) of the fit using:

$$BIC \approx n \log\left(\frac{sse}{n}\right) + K \log(n)$$

where  $n$  is the sample size,  $sse$  is the sum of squared errors of the fit, and  $K$  is the number of parameters in the model<sup>82</sup>. We computed the difference of the BIC scores for each FEN1 tract:

$$\Delta BIC = BIC_{monolinear} - BIC_{bilinear}$$

where larger  $\Delta BIC$  values indicate the FEN1 tract was better fit by the bilinear model. Comparing the  $\Delta BIC$  values across the chases confirmed that the Pol  $\alpha$ -depleted and Pol  $\delta$ -depleted chases were better modeled by the bilinear fitting than the mock chase (data not shown).

### CMG Velocity Analysis

To measure CMG velocity during/after the chase we fit the GINS<sup>LD555</sup> signal with a linear model in MATLAB and extracted the slope. Only timepoints that occurred after the chase were used for fitting.

## REFERENCES

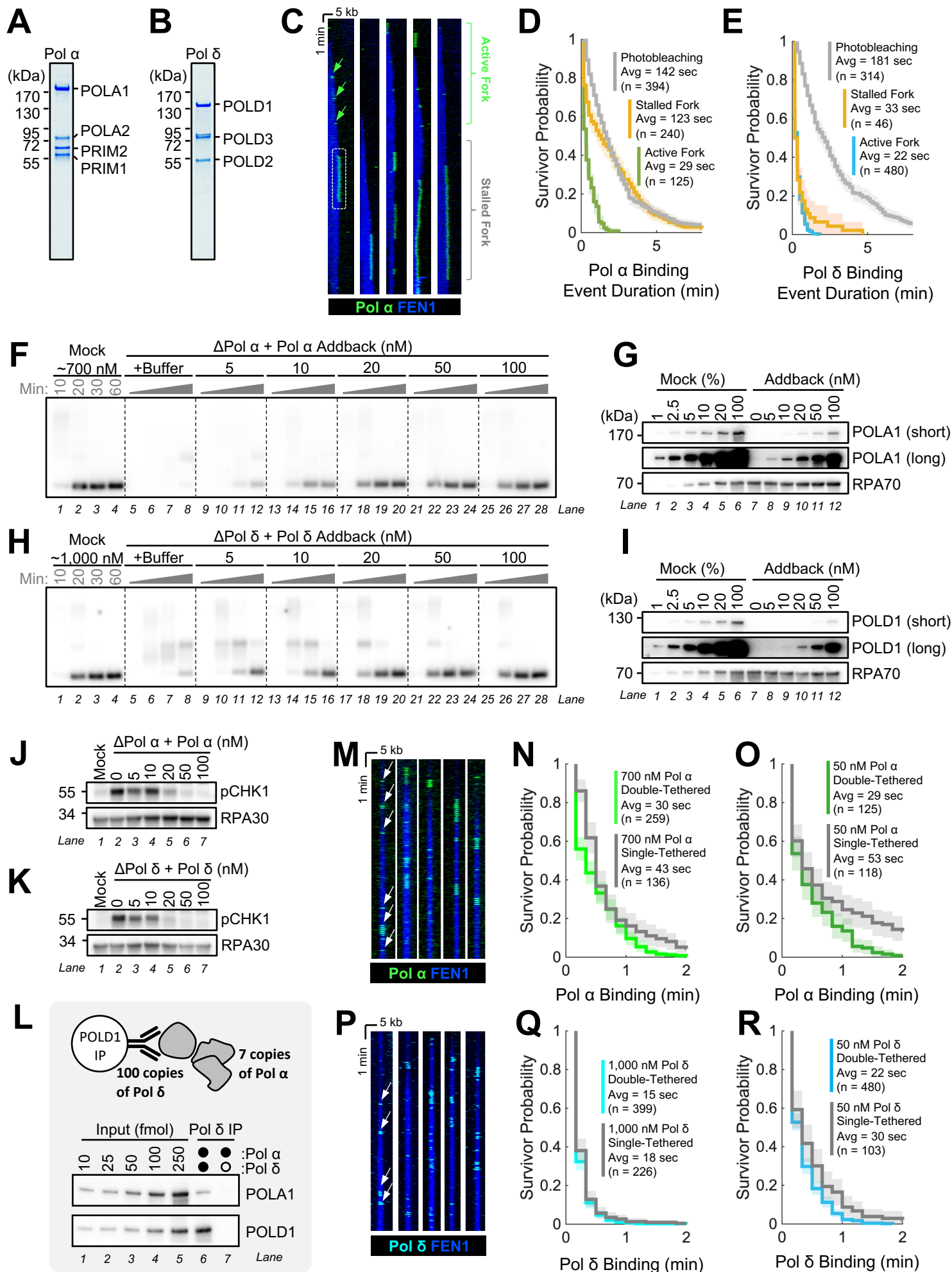
1. Nick McElhinny, S. A., Gordenin, D. A., Stith, C. M., Burgers, P. M. J. & Kunkel, T. A. Division of labor at the eukaryotic replication fork. *Molecular Cell* **30**, 137–144 (2008).
2. Georgescu, R. E. *et al.* Mechanism of asymmetric polymerase assembly at the eukaryotic replication fork. *Nature structural & molecular biology* **21**, 664–670 (2014).
3. Yeeles, J. T. P., Janska, A., Early, A. & Diffley, J. F. X. How the Eukaryotic Replisome Achieves Rapid and Efficient DNA Replication. *Molecular Cell* **65**, 105–116 (2017).
4. Baris, Y., Taylor, M. R. G., Aria, V. & Yeeles, J. T. P. Fast and efficient DNA replication with purified human proteins. *Nature* **606**, 204–210 (2022).
5. Jones, M. L., Aria, V., Baris, Y. & Yeeles, J. T. P. How Pol  $\alpha$ -primase is targeted to replisomes to prime eukaryotic DNA replication. *Molecular Cell* **83**, 2911–2924 (2023).
6. Perera, R. L. *et al.* Mechanism for priming DNA synthesis by yeast DNA Polymerase  $\alpha$ . *eLife* **2**, e00482 (2013).
7. Yuan, Z., Georgescu, R., Li, H. & O'Donnell, M. E. Molecular choreography of primer synthesis by the eukaryotic Pol  $\alpha$ -primase. *Nature Communications* **14**, 3697 (2023).
8. Mullins, E. A. *et al.* A mechanistic model of primer synthesis from catalytic structures of DNA polymerase  $\alpha$ -primase. *Nature Structural & Molecular Biology* **31**, 777–790 (2024).
9. Drosopoulos, W. C., Vierra, D. A., Kenworthy, C. A., Coleman, R. A. & Schildkraut, C. L. Dynamic Assembly and Disassembly of the Human DNA Polymerase  $\delta$  Holoenzyme on the Genome In Vivo. *Cell Reports* **30**, 1329–1341.e5 (2020).
10. Bell, S. P. & Labib, K. Chromosome Duplication in *Saccharomyces cerevisiae*. *Genetics* **203**, 1027–1067 (2016).
11. Langston, L. D. & O'Donnell, M. DNA polymerase delta is highly processive with proliferating cell nuclear antigen and undergoes collision release upon completing DNA. *J Biol Chem* **283**, 29522–29531 (2008).
12. Lupardus, P. J., Byun, T., Yee, M.-C., Hekmat-Nejad, M. & Cimprich, K. A. A requirement for replication in activation of the ATR-dependent DNA damage checkpoint. *Genes Dev* **16**, 2327–2332 (2002).
13. MacDougall, C. A., Byun, T. S., Van, C., Yee, M. & Cimprich, K. A. The structural determinants of checkpoint activation. *Genes Dev* **21**, 898–903 (2007).
14. Saldivar, J. C., Cortez, D. & Cimprich, K. A. The essential kinase ATR: ensuring faithful duplication of a challenging genome. *Nat Rev Mol Cell Biol* **18**, 622–636 (2017).
15. Sinha, N. K., Morris, C. F. & Alberts, B. M. Efficient in vitro replication of double-stranded DNA templates by a purified T4 bacteriophage replication system. *The Journal of Biological Chemistry* **255**, 4290–4293 (1980).
16. Alberts, B. M. *et al.* Studies on DNA replication in the bacteriophage T4 in vitro system. *Cold Spring Harb Symp Quant Biol* **47 Pt 2**, 655–668 (1983).
17. Yao, N. Y. & O'Donnell, M. E. Evolution of replication machines. *Crit Rev Biochem Mol Biol* **51**, 135–149 (2016).
18. Lewis, J. S. *et al.* Tunability of DNA Polymerase Stability during Eukaryotic DNA Replication. *Molecular Cell* **77**, 17–25.e5 (2020).
19. Kapadia, N. *et al.* Processive Activity of Replicative DNA Polymerases in the Replisome of Live Eukaryotic Cells. *Molecular Cell* **80**, 114–126.e8 (2020).
20. Walter, J., Sun, L. & Newport, J. Regulated chromosomal DNA replication in the absence of a nucleus. *Molecular Cell* **1**, 519–529 (1998).
21. Hoogenboom, W. S., Klein Douwel, D. & Knipscheer, P. *Xenopus* egg extract: A powerful tool to study genome maintenance mechanisms. *Developmental Biology* **428**, 300–309 (2017).
22. Yardimci, H., Loveland, A. B., Oijen, A. M. & Walter, J. C. Single-molecule analysis of DNA replication in *Xenopus* egg extracts. *Methods (San Diego, Calif.)* **57**, 179–186 (2012).
23. Loveland, A. B., Habuchi, S., Walter, J. C. & Oijen, A. M. A general approach to break the concentration barrier in single-molecule imaging. *Nature methods* **9**, 987–992 (2012).
24. Kumamoto, S. *et al.* HPF1-dependent PARP activation promotes LIG3-XRCC1-mediated backup pathway of Okazaki fragment ligation. *Nucleic Acids Research* **49**, 5003–5016 (2021).

25. Cho, N. H. *et al.* OpenCell: Endogenous tagging for the cartography of human cellular organization. *Science (New York, N.Y.)* **375**, eabi6983 (2022).
26. Maga, G. & Hübscher, U. DNA replication machinery: functional characterization of a complex containing DNA polymerase alpha, DNA polymerase delta, and replication factor C suggests an asymmetric DNA polymerase dimer. *Biochemistry* **35**, 5764–5777 (1996).
27. Schmid, E. W. & Walter, J. C. Predictomes, a classifier-curated database of AlphaFold-modeled protein-protein interactions. *Molecular Cell* **85**, 1216-1232.e5 (2025).
28. Yardimci, H., Loveland, A. B., Habuchi, S., van Oijen, A. M. & Walter, J. C. Uncoupling of sister replisomes during eukaryotic DNA replication. *Mol Cell* **40**, 834–840 (2010).
29. Liu, Y. *et al.* Fork coupling directs DNA replication elongation and termination. *Science* **383**, 1215–1222 (2024).
30. Hu, Z., Perumal, S. K., Yue, H. & Benkovic, S. J. The human lagging strand DNA polymerase  $\delta$  holoenzyme is distributive. *The Journal of Biological Chemistry* **287**, 38442–38448 (2012).
31. Walter, J. & Newport, J. Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Molecular Cell* **5**, 617–627 (2000).
32. Byun, T. S., Pacek, M., Yee, M., Walter, J. C. & Cimprich, K. A. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes & Development* **19**, 1040–1052 (2005).
33. Baranovskiy, A. G. *et al.* Structural basis for inhibition of DNA replication by aphidicolin. *Nucleic Acids Research* **42**, 14013–14021 (2014).
34. Sparks, J. L. *et al.* The CMG Helicase Bypasses DNA-Protein Cross-Links to Facilitate Their Repair. *Cell* **176**, 167-181.e21 (2019).
35. Han, T. *et al.* The antitumor toxin CD437 is a direct inhibitor of DNA polymerase  $\alpha$ . *Nat Chem Biol* **12**, 511–515 (2016).
36. Klinge, S., Hirst, J., Maman, J. D., Krude, T. & Pellegrini, L. An iron-sulfur domain of the eukaryotic primase is essential for RNA primer synthesis. *Nature Structural & Molecular Biology* **14**, 875–877 (2007).
37. Weiner, B. E. *et al.* An iron-sulfur cluster in the C-terminal domain of the p58 subunit of human DNA primase. *The Journal of Biological Chemistry* **282**, 33444–33451 (2007).
38. Netz, D. J. A. *et al.* Eukaryotic DNA polymerases require an iron-sulfur cluster for the formation of active complexes. *Nature Chemical Biology* **8**, 125–132 (2011).
39. Shaw, A. E. *et al.* Revised mechanism of hydroxyurea-induced cell cycle arrest and an improved alternative. *Proceedings of the National Academy of Sciences of the United States of America* **121**, e2404470121 (2024).
40. Yuan, Z. *et al.* Structure of the eukaryotic replicative CMG helicase and pumpjack motion. *Nature structural & molecular biology* **23**, 217–224 (2016).
41. Lancey, C. *et al.* Structure of the processive human Pol  $\delta$  holoenzyme. *Nature Communications* **11**, 1109 (2020).
42. Huis in 't Veld, P. J. *et al.* Characterization of a DNA exit gate in the human cohesin ring. *Science (New York, N.Y.)* **346**, 968–972 (2014).
43. Maloisel, L., Fabre, F. & Gangloff, S. DNA Polymerase  $\delta$  Is Preferentially Recruited during Homologous Recombination To Promote Heteroduplex DNA Extension. *Molecular and Cellular Biology* **28**, 1373–1382 (2008).
44. Donnianni, R. A. *et al.* DNA Polymerase Delta Synthesizes Both Strands during Break-Induced Replication. *Molecular Cell* **76**, 371-381.e4 (2019).
45. Meyer, D., Fu, B. X. H. & Heyer, W.-D. DNA polymerases  $\delta$  and  $\lambda$  cooperate in repairing double-strand breaks by microhomology-mediated end-joining in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **112**, E6907-6916 (2015).
46. Engel, S. R. *et al.* *Saccharomyces* Genome Database: advances in genome annotation, expanded biochemical pathways, and other key enhancements. *Genetics* **229**, iyae185 (2025).
47. Porcella, S. Y. *et al.* Separable, Ctf4-mediated recruitment of DNA Polymerase  $\alpha$  for initiation of DNA synthesis at replication origins and lagging-strand priming during replication elongation. *PLoS Genet* **16**, e1008755 (2020).

48. Koussa, N. C. & Smith, D. J. Limiting DNA polymerase delta alters replication dynamics and leads to a dependence on checkpoint activation and recombination-mediated DNA repair. *PLoS genetics* **17**, e1009322 (2021).
49. Hedglin, M., Pandey, B. & Benkovic, S. J. Stability of the human polymerase  $\delta$  holoenzyme and its implications in lagging strand DNA synthesis. *Proc Natl Acad Sci U S A* **113**, E1777-1786 (2016).
50. Zheng, F., Georgescu, R. E., Li, H. & O'Donnell, M. E. Structure of eukaryotic DNA polymerase  $\delta$  bound to the PCNA clamp while encircling DNA. *Proc Natl Acad Sci U S A* **117**, 30344–30353 (2020).
51. Podust, V. N., Podust, L. M., Müller, F. & Hübscher, U. DNA polymerase delta holoenzyme: action on single-stranded DNA and on double-stranded DNA in the presence of replicative DNA helicases. *Biochemistry* **34**, 5003–5010 (1995).
52. Masuda, Y. *et al.* Dynamics of human replication factors in the elongation phase of DNA replication. *Nucleic Acids Res* **35**, 6904–6916 (2007).
53. Raducanu, V.-S. *et al.* Mechanistic investigation of human maturation of Okazaki fragments reveals slow kinetics. *Nat Commun* **13**, 6973 (2022).
54. Wu, C. A. *et al.* Coordinated leading- and lagging-strand synthesis at the Escherichia coli DNA replication fork. IV. Reconstitution of an asymmetric, dimeric DNA polymerase III holoenzyme. *J Biol Chem* **267**, 4064–4073 (1992).
55. Kim, S., Dallmann, H. G., McHenry, C. S. & Marians, K. J. tau couples the leading- and lagging-strand polymerases at the Escherichia coli DNA replication fork. *J Biol Chem* **271**, 21406–21412 (1996).
56. Yang, J., Zhuang, Z., Roccasacca, R. M., Trakselis, M. A. & Benkovic, S. J. The dynamic processivity of the T4 DNA polymerase during replication. *Proc Natl Acad Sci U S A* **101**, 8289–8294 (2004).
57. Johnson, D. E., Takahashi, M., Hamdan, S. M., Lee, S.-J. & Richardson, C. C. Exchange of DNA polymerases at the replication fork of bacteriophage T7. *Proc Natl Acad Sci U S A* **104**, 5312–5317 (2007).
58. Loparo, J. J., Kulczyk, A. W., Richardson, C. C. & van Oijen, A. M. Simultaneous single-molecule measurements of phage T7 replisome composition and function reveal the mechanism of polymerase exchange. *Proc Natl Acad Sci U S A* **108**, 3584–3589 (2011).
59. Liao, Y., Li, Y., Schroeder, J. W., Simmons, L. A. & Biteen, J. S. Single-Molecule DNA Polymerase Dynamics at a Bacterial Replisome in Live Cells. *Biophys J* **111**, 2562–2569 (2016).
60. Beattie, T. R. *et al.* Frequent exchange of the DNA polymerase during bacterial chromosome replication. *eLife* **6**, e21763 (2017).
61. Lewis, J. S. *et al.* Single-molecule visualization of fast polymerase turnover in the bacterial replisome. *Elife* **6**, e23932 (2017).
62. Åberg, C., Duderstadt, K. E. & van Oijen, A. M. Stability versus exchange: a paradox in DNA replication. *Nucleic Acids Res* **44**, 4846–4854 (2016).
63. Cseresnyes, Z., Schwarz, U. & Green, C. M. Analysis of replication factories in human cells by super-resolution light microscopy. *BMC Cell Biol* **10**, 88 (2009).
64. Hawgood, M. *et al.* Spatial mapping of DNA synthesis reveals dynamics and geometry of human replication nanostructures. *EMBO J* **44**, 7263–7294 (2025).
65. Lee, J.-B. *et al.* DNA primase acts as a molecular brake in DNA replication. *Nature* **439**, 621–624 (2006).
66. Burgers, P. M. J. & Kunkel, T. A. Eukaryotic DNA Replication Fork. *Annual Review of Biochemistry* **86**, 417–438 (2017).
67. Gan, H. *et al.* Checkpoint kinase Rad53 couples leading and lagging strand DNA synthesis under replication stress. *Molecular cell* **68**, 446–455.e3 (2017).
68. Bainbridge, L. J. & Daigaku, Y. Bulk synthesis and beyond: The roles of eukaryotic replicative DNA polymerases. *DNA Repair (Amst)* **141**, 103740 (2024).
69. Ercilla, A. *et al.* Physiological Tolerance to ssDNA Enables Strand Uncoupling during DNA Replication. *Cell Rep* **30**, 2416–2429.e7 (2020).
70. Mehta, K. P. M. *et al.* CHK1 phosphorylates PRIMPOL to promote replication stress tolerance. *Science Advances* **8**, eabm0314 (2022).
71. Rodríguez-Acebes, S. *et al.* DNA polymerase  $\alpha$ /primase extraction from chromatin by VCP/p97 restricts ATR activation during unperturbed DNA replication. *Nat Commun* **16**, 5706 (2025).

72. Graham, J. E., Marians, K. J. & Kowalczykowski, S. C. Independent and Stochastic Action of DNA Polymerases in the Replisome. *Cell* **169**, 1201-1213.e17 (2017).
73. Marians, K. J. Lesion Bypass and the Reactivation of Stalled Replication Forks. *Annu Rev Biochem* **87**, 217–238 (2018).
74. Stano, N. M. *et al.* DNA synthesis provides the driving force to accelerate DNA unwinding by a helicase. *Nature* **435**, 370–373 (2005).
75. Berger, S. & Chistol, G. Visualizing the dynamics of DNA replication and repair at the single-molecule level. *Methods Cell Biol* **182**, 109–165 (2024).
76. Lebofsky, R., Takahashi, T. & Walter, J. C. DNA replication in nucleus-free *Xenopus* egg extracts. *Methods Mol Biol* **521**, 229–252 (2009).
77. Weissmann, F. *et al.* biGBac enables rapid gene assembly for the expression of large multisubunit protein complexes. *Proc Natl Acad Sci U S A* **113**, E2564-2569 (2016).
78. Sari, D. *et al.* The MultiBac Baculovirus/Insect Cell Expression Vector System for Producing Complex Protein Biologics. *Adv Exp Med Biol* **896**, 199–215 (2016).
79. Modesti, M. Fluorescent labeling of proteins. *Methods Mol Biol* **783**, 101–120 (2011).
80. Yin, J. *et al.* Genetically encoded short peptide tag for versatile protein labeling by Sfp phosphopantetheinyl transferase. *Proc Natl Acad Sci U S A* **102**, 15815–15820 (2005).
81. Terui, R., Berger, S. E., Sambel, L. A., Song, D. & Chistol, G. Single-molecule imaging reveals the mechanism of bidirectional replication initiation in metazoa. *Cell* **187**, 3992-4009.e25 (2024).
82. Cavanaugh, J. E. & Neath, A. A. The Akaike information criterion: Background, derivation, properties, application, interpretation, and refinements. *WIREs Computational Statistics* **11**, e1460 (2019).

# Figure S1 (related to Figure 1)

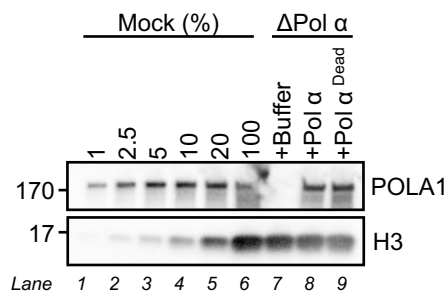


### Figure S1: Validation of Recombinant Pol $\alpha/\delta$ and Other Reagents

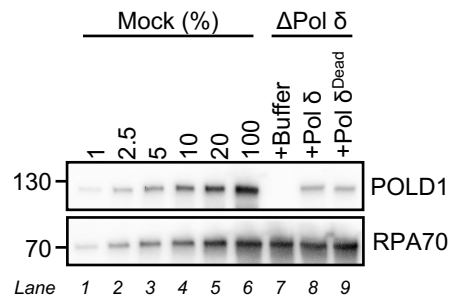
(A) Coomassie gel of purified recombinant Pol  $\alpha$  used in this study. (B) Coomassie gel of purified recombinant Pol  $\delta$  used in this study. (C) Five representative kymograms Pol  $\alpha^{AF647}$  (green) signal at active (green arrows) and stalled (white box) replication forks. (D) Survivor probability curves for Pol  $\alpha^{AF647}$  binding event duration at active forks (green), stalled forks (orange), and photobleaching controls (gray). Note that the duration of Pol  $\alpha^{AF647}$  binding events at stalled forks is limited by photobleaching, suggesting that Pol  $\alpha^{AF647}$  resides at stalled forks longer than the estimated 123 seconds. (E) Survivor probability curves of Pol  $\delta^{AF647}$  signal duration at active forks (green), stalled forks (orange), and photobleaching controls (gray). (F) Replication assay depicting Pol  $\alpha$  titration. (G) POLA1 immunoblot corresponding to the experiment in panel (F). (H) Replication assay depicting Pol  $\delta$  titration. (I) POLD1 immunoblot corresponding to the experiment in panel (H). (J) pCHK1 immunoblot for the Pol  $\alpha$  titration experiment in panel (F). (K) pCHK1 immunoblot for the Pol  $\delta$  titration experiment in panel (H). (L) Co-immunoprecipitation experiment of Pol  $\alpha$  with Pol  $\delta$ . We estimated that for every  $\sim 100$  molecules of immunoprecipitated Pol  $\delta$ , we co-IP  $\sim 7$  molecules of Pol  $\alpha$ . (M) Five representative kymograms of Pol  $\alpha^{AF647}$  (green) binding to single-tethered DNA molecules that are condensed in a diffraction-limited spot. The accumulation of FEN1<sup>mKikGR</sup> signal (blue) indicates that these DNA molecules are replicated. (N) Survivor probability curves for Pol  $\alpha^{AF647}$  binding event duration on single-tethered DNA (gray) versus double-tethered DNA (green) from an experiment where 20 nM of Pol  $\alpha^{AF647}$  was spiked into undepleted extract ( $\sim 700$  nM of endogenous Pol  $\alpha$ ). (O) Same as in panel (N), but for an experiment where 5 nM of Pol  $\alpha^{AF647}$  was spiked into Pol  $\alpha$ -depleted extract supplemented with 45 nM of recombinant Pol  $\alpha$  (50 nM of total Pol  $\alpha$ ). (P) Five representative kymograms of Pol  $\delta^{AF647}$  (cyan) and FEN1<sup>mKikGR</sup> (blue) binding to single-tethered DNA molecules. (Q) Survivor probability curves for Pol  $\delta^{AF647}$  binding event duration on single-tethered DNA (gray) versus double-tethered DNA (cyan) from an experiment where 20 nM of Pol  $\delta^{AF647}$  was spiked into undepleted extract ( $\sim 1000$  nM of endogenous Pol  $\delta$ ). (R) Same as in panel (Q), but for an experiment where 5 nM of Pol  $\delta^{AF647}$  was spiked into Pol  $\delta$ -depleted extract supplemented with 45 nM of recombinant Pol  $\delta$  (50 nM of total Pol  $\delta$ ). In panels (D), (E), (N), (O), (Q), and (R) semi-transparent boxes depict the 95% Confidence Interval (CI) estimated via bootstrapping; 'n' denotes the number of binding events analyzed.

# Figure S2 (related to Figure 2)

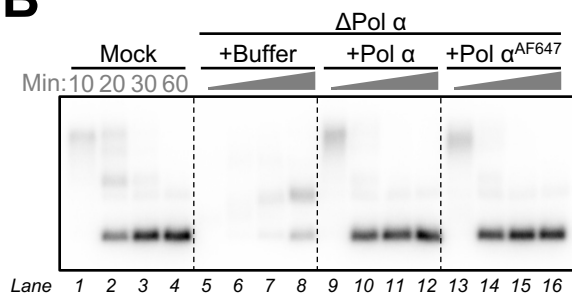
**A**



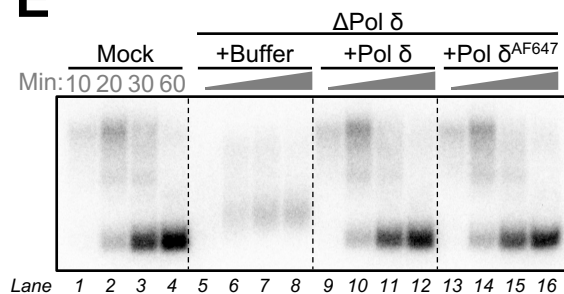
**D**



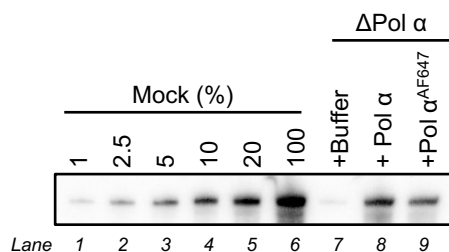
**B**



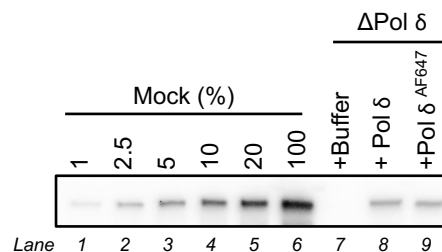
**E**



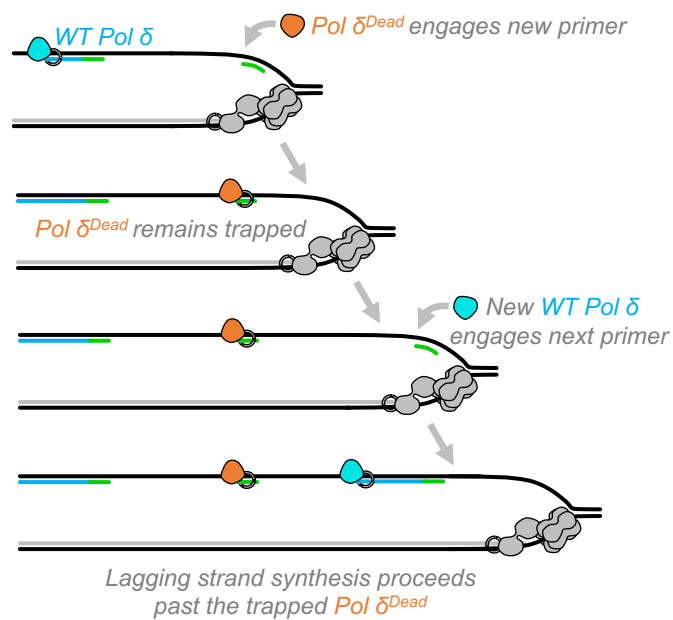
**C**



**F**



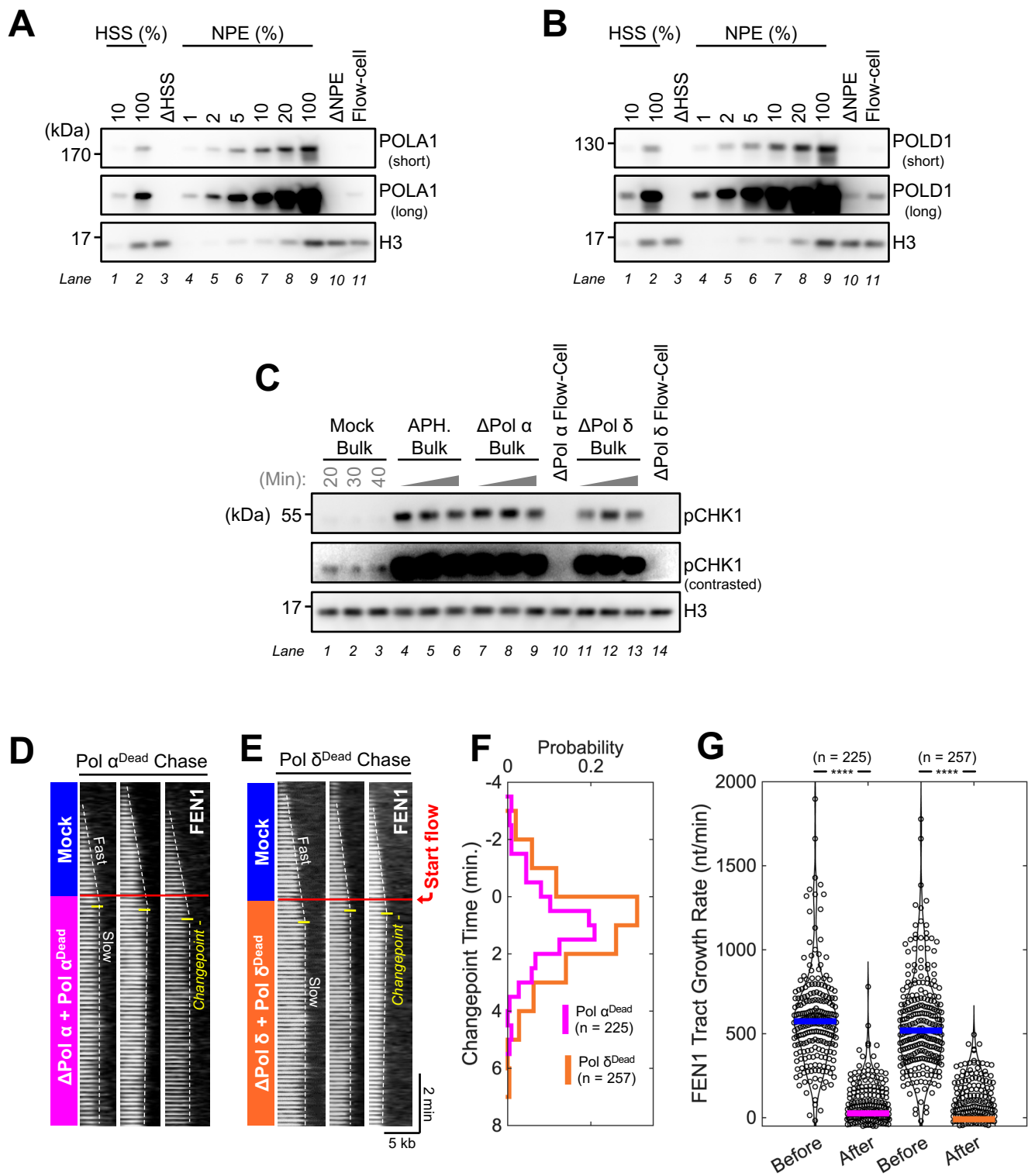
**G**



**Figure S2: Validation of Catalytically Dead Pol  $\alpha/\delta$  Recombinant Proteins**

(A) POLA1 immunoblot corresponding to the experiment from Figure 2B. (B) Ensemble plasmid replication assay comparing the ability of wildtype Pol  $\alpha$  and Pol  $\alpha^{\text{AF647}}$  to support DNA replication in Pol  $\alpha$ -depleted extract. (C) POLA1 immunoblot corresponding to the experiment in panel (B). (D) POLD1 immunoblot corresponding to the experiment from Figure 2F. (E) Ensemble plasmid replication assay comparing the ability of wildtype Pol  $\delta$  and Pol  $\delta^{\text{AF647}}$  to support DNA replication in Pol  $\delta$ -depleted extract. (F) POLD1 immunoblot corresponding to the experiment in panel (E). (G) Cartoon illustrating Pol  $\delta^{\text{Dead}}$  being trapped behind an advancing replication fork.

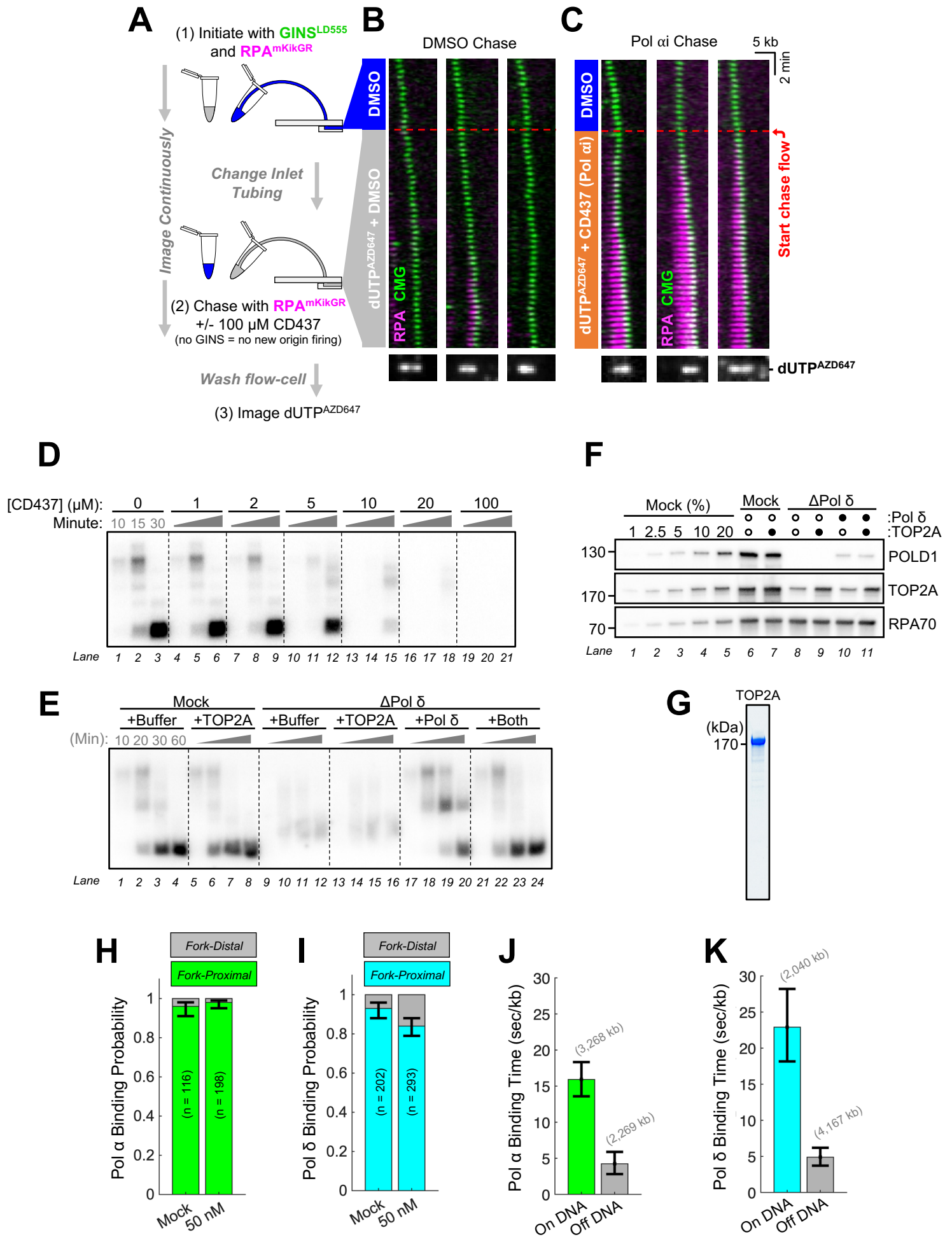
# Figure S3 (related to Figure 3)



### Figure S3: Validation of Polymerase-Depleted Chase Experiments

(A) POLA1 immunoblot corresponding to the experiment from Figure 3D. (B) POLD1 immunoblot corresponding to the experiment from Figure 3G. (C) pCHK1 immunoblot for the experiments from Figure 3D and Figure 3G. Samples were compared to bulk ensemble mock (lanes 1-3), 5  $\mu$ M aphidicolin treated (lanes 4-6), Pol  $\alpha$ -depleted (lanes 7-9), or Pol  $\delta$ -depleted (lanes 11-13) replication reactions. (D) Three representative kymograms from the chase with Pol  $\alpha$ -depleted extract supplemented with 500 nM Pol  $\alpha^{\text{Dead}}$ . The dashed line marks the edge of the FEN1 tract fit by two straight lines with a changepoint (marked in yellow). (E) Three representative kymograms from the chase with Pol  $\delta$ -depleted extract supplemented with 500 nM Pol  $\delta^{\text{Dead}}$ . (F) Time of the changepoint measured for the Pol  $\alpha^{\text{Dead}}$  chase (magenta) and the Pol  $\delta^{\text{Dead}}$  chase (orange). Zero denotes the start of the chase flow. (G) The growth rate of the FEN1<sup>mKikGR</sup> tract before and after the chase with Pol  $\alpha^{\text{Dead}}$  (magenta) or Pol  $\delta^{\text{Dead}}$  (orange). In panels (F) and (G) 'n' denotes the number of replication forks analyzed. In panel (G) '\*\*\*\*' denotes  $p < 0.0001$ . p-values were computed using the two-sample Kolmogorov-Smirnov test. Semi-transparent boxes represent the 95% CI estimated via bootstrapping.

# Figure S4 (related to Figure 4)



#### Figure S4: Additional Controls and Reagent Validation

(A) Diagram of the single-molecule chase experiment with dUTP<sup>AZD647</sup>. (B) Three representative kymograms of CMG (green) and RPA<sup>mKikGR</sup> (magenta) from an experiment where the chase extract contained DMSO and 16.7  $\mu$ M dUTP<sup>AZD647</sup>. CMG was labeled by replacing endogenous GINS with fluorescently-labeled GINS<sup>LD555</sup>. The red dashed line marks the start of the chase flow. The panel below each kymogram shows the dUTP<sup>AZD647</sup> signal imaged at the end of the experiment: the white tract represents dUTP<sup>AZD647</sup> incorporated into nascent DNA during the chase. (C) Three representative kymograms from an experiment where the chase extract contained 100  $\mu$ M CD437 (Pol  $\alpha$  inhibitor) and 16.7  $\mu$ M dUTP<sup>AZD647</sup>. Note that the Pol  $\alpha$  inhibitor did not prevent dUTP<sup>AZD647</sup> incorporation into nascent DNA, indicating that the dUTP<sup>AZD647</sup> signal is derived from leading strand synthesis. (D) Titration of CD437 in an ensemble plasmid replication assay. Note that only CD437 concentrations above 20  $\mu$ M fully ablate DNA synthesis. (E) Ensemble plasmid replication assay where Pol  $\delta$ -depleted extract was supplemented with 200 nM recombinant TOP2A, 100 nM recombinant Pol  $\delta$ , or both. (F) Immunoblot of POLD1 and TOP2A corresponding to the experiment from panel (E). (G) Coomassie gel of purified recombinant TOP2A used in this study. (H) Probability of Pol  $\alpha^{AF647}$  binding near the fork (fork-proximal) or far away from the fork (fork-distal). Data analyzed from experiments shown in Figure 1C and Figure 1G. (I) Probability of Pol  $\delta^{AF647}$  binding near the fork (fork-proximal) or far away from the fork (fork-distal). Data analyzed from experiments shown in Figure 1E and Figure 1H. (J) Total binding time of Pol  $\alpha^{AF647}$  binding events measured on replicating DNA versus non-specific Pol  $\alpha^{AF647}$  binding to areas of the coverslip that contained no DNA. Data analyzed from the experiment shown Figure 1G. This analysis indicates that the majority of Pol  $\alpha^{AF647}$  binding events occur specifically along replicating DNA. (K) Total binding time of Pol  $\delta^{AF647}$  binding events measured on replicating DNA versus non-specific Pol  $\delta^{AF647}$  binding to areas of the coverslip that contained no DNA. Data analyzed from the experiment shown Figure 1H. This analysis indicates that the majority of Pol  $\delta^{AF647}$  binding events occur specifically along replicating DNA. In panels (H), (I), (J), and (K) error bars depict the 95% CI estimated via bootstrapping. In panels (H) and (I), 'n' denotes the number of binding events analyzed. In panels (J) and (K), the number in parentheses represents the total length of DNA analyzed (for reference, each  $\lambda$  DNA molecule is ~48 kb long).