A gatekeeping function of the replicative polymerase controls pathway choice in the resolution of lesion-stalled replisomes

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Abstract
DNA lesions stall the replisome and proper resolution of these obstructions is critical for genome stability. Replisomes can directly replicate past a lesion by error-prone translesion synthesis. Alternatively, replisomes can reprim DNA synthesis downstream of the lesion, creating a single-stranded DNA gap that is repaired primarily in an error-free, homology-directed manner. Here we demonstrate how structural changes within the Escherichia coli replisome determine the resolution pathway of lesion-stalled replisomes. This pathway selection is controlled by a dynamic interaction between the proofreading subunit of the replicative polymerase and the processivity clamp, which sets a kinetic barrier to restrict access of translesion synthesis (TLS) polymerases to the primer/template junction. Failure of TLS polymerases to overcome this barrier leads to repriming, which competes kinetically with TLS. Our results demonstrate that independent of its exonuclease activity, the proofreading subunit of the replisome acts as a gatekeeper and influences replication fidelity during the resolution of lesion-stalled replisomes.

Publication Details

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This journal article is available at Research Online: https://ro.uow.edu.au/smhpapers1/1071
A gatekeeping function of the replicative polymerase controls pathway choice in the resolution of lesion-stalled replisomes

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Edited by Michael E. O’Donnell, HHMI and The Rockefeller University, New York, NY, and approved November 5, 2019 (received for review August 21, 2019)

DNA lesions stall the replisome and proper resolution of these obstructions is critical for genome stability. Replisomes can directly replicate past a lesion by error-prone translesion synthesis. Alternatively, replisomes can reprogram DNA synthesis downstream of the lesion, creating a single-stranded DNA gap that is repaired primarily in an error-free, homology-directed manner. Here we demonstrate how structural changes within the *Escherichia coli* replisome determine the resolution pathway of lesion-stalled replisomes. This pathway selection is controlled by a dynamic interaction between the proofreading subunit of the replicative polymerase and the processivity clamp, which sets a kinetic barrier to restrict access of translesion synthesis (TLS) polymerases to the primer/template junction. Failure of TLS polymerases to overcome this barrier leads to repriming, which competes kinetically with TLS. Our results demonstrate that independent of its exonuclease activity, the proofreading subunit of the replisome acts as a gatekeeper and influences replication fidelity during the resolution of lesion-stalled replisomes.

DNA replication | replication stalling | translesion synthesis | damage avoidance | reprogramming

**Genomic DNA is constantly damaged by various intracellular and extracellular agents. Replication is transiently blocked at these sites because replicative DNA polymerases are generally poor at synthesizing past lesions. Stalled replisomes are resolved primarily through either recombination-dependent damage avoidance (DA) pathways or translesion synthesis (TLS), and distinct DNA intermediates are created during each process (1). In DA pathways, damaged templates are implicated in an error-free manner using an undamaged homologous sister chromatid as a template via processes that utilize homologous recombination factors. In contrast, during TLS damaged templates are directly replicated by TLS polymerases in an error-prone manner. TLS can occur through sequential polymerase switching between the replicative polymerase and a TLS polymerase, yielding a continuous DNA product (TLS at the fork). Alternatively, replisomes can reprogram DNA synthesis downstream of the lesion leaving a single-stranded DNA (ssDNA) gap behind. These gaps are then filled in either by TLS polymerases (gap-filling synthesis) or by homology-dependent gap repair (HDGR) (1). Given the marked differences in mutagenic potential between DA pathways and TLS, it is important to understand what determines pathway choice at stalled replisomes. Structural changes within the replisome upon lesion stalling, such as conformational changes of replisome components and alterations in protein–protein interactions, likely play an important role in pathway selection, yet these dynamics have been largely unexplored. DNA replication of *Escherichia coli* serves as an attractive model system to probe these lesion-induced structural changes of the replisome as it uses both DA pathways and TLS to resolve stalled replisomes. Moreover, the *E. coli* replisome can be reconstituted with a relatively small number of factors and is genetically tractable while retaining the same basic architecture of more complicated systems (2, 3). In *E. coli* the majority of lesion-stalled replisomes are resolved through DA pathways, particularly when the SOS damage response is not induced. However, upon induction of the SOS response and the concomitant increase in TLS polymerase levels, higher fractions of stalled replisomes are resolved through TLS (4, 5).

**E. coli** cells have 3 TLS polymerases, Pol II, IV, and V (5, 6). Among these, Pol II and IV are abundant even before their expression levels are highly elevated during the damage-induced SOS response. If TLS polymerases gained frequent access to the extending primer, replication would be severely inhibited due to the much slower polymerization of TLS polymerases as compared to Pol III, the replicative polymerase (7–9). Intriguingly, despite the high abundance of Pol II and Pol IV relative to the replicative polymerase both in the SOS response-uninduced and -induced cells, TLS polymerases only modestly inhibit replication.

**Significance**

DNA replication is the high-fidelity process by which cells duplicate their chromosomes prior to cell division. Cellular DNA is constantly damaged, and the resulting DNA lesions can block replication, leading to genome instability and cell death. Cells use multiple pathways to resolve stalled replication. Understanding resolution pathway choice is important because some of these pathways are more likely to introduce mutations than others. In bacterial cells, replication stresses, including antibiotic treatment, lead to mutagenesis, which may contribute to the emergence of antibiotic resistance. In this study, we describe a molecular interaction within the *Escherichia coli* replication machinery that plays a crucial role in resolution pathway choice, thereby influencing whether lesion-stalled replication is resolved in an error-prone or error-free manner.


The authors declare no competing interest.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1914485116/-DCSupplemental.

First published December 3, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1914485116
in *E. coli* cells (10) and they contribute little to spontaneous mutagenesis (11, 12). Collectively, these observations suggest that TLS polymerases are largely excluded from replisomes (13, 14).

The *E. coli* β2 clamp processivity factor plays an important role in regulating TLS because all TLS polymerases must bind the β2 clamp to perform TLS (5, 9, 15–17). The β2 clamp is a homodimer that encircles DNA and tethers DNA polymerases to their template (18, 19). Each protomer of the β2 clamp molecule has a hydrophobic cleft, a common binding site for clamp binding proteins, yielding 2 identical clefts per β2 clamp molecule (20).

Clamp binding proteins have 1 or more clamp binding motifs (CBMs) that interact with the β2 clamp via cleft–CBM interactions (21, 22). All 5 *E. coli* DNA polymerases have 1 or 2 conserved CBMs (17, 21, 23). Pol III complex is a trimeric complex (αβθ) consisting of polymerase (α), exonuclease (ε) and accessory (θ) subunits. The α subunit has an internal CBM, which is required for processive replication (24). In addition, the ε subunit has an internal CBM, which is responsible for the replication-promoting role of the ε subunit (25–27). During processive replication, the α and ε subunits of the Pol III complex occupy both clefts of a β2 clamp molecule (26–28). However, unlike the internal CBM of the α subunit, the CBM of the ε subunit has a relative low binding affinity to the cleft; this results in its frequent detachment from a cleft, causing temporary pauses during processive replication (29, 30). Given the dynamic nature of the ε–β2 clamp interaction, it may constitute a key factor in regulating the access of cleft-binding proteins, such as TLS polymerases.

Here we show that dynamic interactions between Pol III and the β2 clamp dictate the fate of lesion-stalled replisomes. We demonstrate that when Pol IV is present at optimal levels, TLS occurs at the fork through sequential polymerase switching between Pol III and Pol IV. In contrast, when Pol IV is present at suboptimal levels, a higher fraction of lesion-stalled replisomes is resolved by repriming replication downstream, leaving an ssDNA gap. We show that besides its canonical proofreading function, the ε subunit plays a gatekeeping role that largely prevents Pol IV from replacing Pol III during processive replication and limits the usage of TLS to resolve lesion-stalled replisomes. Central to this gatekeeping role is the dynamic interaction between the ε subunit and the β2 clamp. When this interaction is strengthened in vitro or in Pol IV-mediated TLS at the fork is suppressed, leading to more lesion skipping. Conversely, when the ε–β2 clamp interaction is weakened, Pol IV more efficiently mediates TLS at the fork and processive replication is more readily inhibited by Pol IV. In cells, when the SOS-response is not induced, TLS by all 3 TLS polymerases is minimally employed to resolve lesion-stalled replisomes. However, weakening the ε–β2 clamp interaction leads to resolution of a much larger fraction of lesion-stalled replisomes through TLS, indicating that the strength of the ε–β2 interaction is tuned to suppress TLS in uninduced cells. Collectively, these results show that the ε–β2 interaction sets a kinetic barrier to the access of TLS polymerases to the extending primer and thus suppresses TLS.

**Results**

**Rapid and Efficient Pol IV-Mediated TLS within Lesion-Stalled Replisomes.** In an effort to determine how processive replication and TLS are coordinated, we reconstituted Pol IV-mediated TLS on a rolling-circle DNA template that contains a site-specific N2-furfuryl dG (N2-FFdG) lesion on the leading-strand template (Fig. L4 and *SI Appendix*, Fig. S1L4). N2-FFdG is an attractive model lesion because Pol IV is proficient at replicating past N2-dG adducts and structurally related DNA lesions can be created in living cells by treatment with nitrofurazone (NFDZ) (31). In rolling-circle replication, replication proceeds over the circular template multiple times, generating a long leading-strand tail that serves as a template for discontinuous lagging-strand synthesis (Fig. L4 (2, 32, 33). Replication of the lesion-free control template by the reconstituted *E. coli* replisome resulted in the rapid formation of replication products that can be resolved by denaturing gel electrophoresis: A resolution-limited leading-strand product band and a distribution of smaller lagging-strand products along with a fraction of unreplicated templates (Fig. 1B and *SI Appendix*, Fig. S1B) (2). The resolution limit (RL) of our gel is ~45 kilonucleotides, and therefore accumulation of leading-strand products at the RL indicates that at least 6 cycles of rolling-circle replication occurred on each template. Consistent with a prior observation that a single N2-FFdG blocks primer extension by Pol III (9), replication of the N2-FFdG-containing template in the absence of Pol IV was strongly attenuated by a single N2-FFdG on the leading-strand template (Fig. 1B). We also observed faint, discrete bands between resolution-limited replication products and unreplicated templates (Fig. 1 B and C). As these products were created only in the presence of N2-FFdG, they represent lesion-stalled replisomes that have undergone different multiples of replication around the template with TLS over the lesion inefficiently mediated by Pol III (34).

To determine whether Pol IV-mediated TLS might resolve this stalling, we examined the effect of Pol IV on replication of the N2-FFdG-containing template. Upon addition of increasing amounts of Pol IV, synthesis of both the leading and lagging strands was gradually restored and long leading-strand replication products accumulated at the RL before replication was fully inhibited by Pol IV at high concentrations (Fig. 1C and *SI Appendix*, Fig. S1E) (7). The robust formation of these resolution-limited products shows that TLS occurs efficiently over the lesion at the fork as repriming of leading-strand synthesis would result in a gap that terminates rolling circle replication. This termination results from the displacement of the circular leading-strand template that occurs when the helicase runs into the strand discontinuity resulting from repriming (*SI Appendix*, Fig. S1F).

Consistent with the requirements of Pol IV-mediated TLS in cells, the ability of Pol IV to promote replication of the N2-FFdG-containing template required both its catalytic and clamp-binding activities (*SI Appendix*, Fig. S1 D, E, and G) (17). Pol IV-mediated TLS occurred less efficiently over an N2-(1-carboxyethyl)-2-deoxyguanosine (N2–CEdG) lesion and was strongly blocked by tetrahydrofuran (THF) (*SI Appendix*, Fig. S1F), indicating that our rolling-circle assay is sensitive to the efficiency by which Pol IV mediates TLS over various lesions (35, 36).

Moreover, at optimal Pol IV concentrations, replication of the N2-FFdG-containing template, as measured by quantifying leading-strand replication products (*SI Appendix*, Fig. S1C), was slowed by only ~40% compared to replication of the control template (*SI Appendix*, Fig. S1F). Given that the polymerization rate of the Pol IV-based *E. coli* replisome is ~10 nts/sec (7, 9), which is over 50 times lower than that of the Pol III-based replisome, these results indicate that Pol IV only briefly switches with Pol III (9, 37). Collectively, these results demonstrate that within our reconstitution, Pol IV can efficiently mediate TLS at the replication fork by switching with Pol III, synthesizing a small patch of DNA over the lesion.

The ε–Cleft Contact Mediates Polymerase Exchange during TLS. As Pol III core (αθ) occupancy both clefts of the β2 clamp during processive replication (26) and Pol IV must bind to a cleft for its action, we sought to determine how Pol III disengages from the clamp to allow for Pol IV to mediate TLS at the fork. To address this question, we varied the strength of the interaction between the Pol III core and the β2 clamp and examined the effect on TLS. The α and ε subunits of the Pol III core (αθ) each occupy a cleft via independent CBMs, albeit with vastly different affinities; the ε–cleft interaction is ~250-fold weaker than the α–cleft interaction (Fig. 24) (24, 26). Replacing the wild-type CBM of the ε subunit with a mutant CBM (ε3), which binds the cleft ~500
times tighter than the wild-type CBM (26), suppressed Pol IV-mediated TLS compared with the wild-type Pol III core (αδθ) and required higher Pol IV concentrations for optimal TLS (Fig. 2B). Normalizing replication of the lesion-containing template by replication of the lesion-free control template (SI Appendix, Supplementary Methods) showed that the εL mutation suppressed Pol IV-mediated TLS to ∼40% (Fig. 2B, Right Inset). Strengthening the interaction also modestly enhanced replicative activity on a lesion-free template (Fig. 2B, Left Inset) (26). These results suggest that the disengagement of the ε subunit from the β2 clamp at a lesion is necessary for Pol IV to bind to the β2 clamp and perform TLS.

As the α subunit also contains a CBM, we next considered if disengagement of α was additionally required for Pol IV-mediated TLS (Fig. 2A). To address this possibility, we examined the effect of strengthening the α-clamp interaction within our biochemical reconstitution. Unlike the effects of strengthening the ε-clamp interaction, replacing the wild-type CBM of the α subunit with a mutant CBM (αδθεM3), which binds the clamp ∼100 times tighter than the wild-type CBM (24)—even ∼50 times tighter than the εL-clamp interaction—had little effect on replication of the lesion-free or lesion-containing template (Fig. 2C). Collectively, these results demonstrate that disengagement of only the ε subunit, not the α subunit, within lesion-stalled replisomes promotes Pol IV to bind to a clamp and perform TLS.

To validate these in vitro observations in cells, we introduced the dnaQ(εL) and dnaE(αεM3) mutations, individually or in combination, into their respective genomic loci and examined their effects on cellular sensitivity to DNA damaging agents NFZ and methyl methanesulfonate (MMS) (Fig. 2D). Cells lacking Pol IV (ΔdinB) were strongly sensitized to NFZ and MMS, indicating that Pol IV-mediated TLS contributes to cellular tolerance to these agents. Consistent with our in vitro observations, the dnaQ(εL) strain was sensitized to both NFZ and MMS, whereas the dnaE(αεM3) strain retained wild-type tolerance. Furthermore, the strain containing both the dnaQ(εL) and dnaE(αεM3) mutations [dnaQ(εL) dnaE(αεM3)] resembled the sensitivity of the dnaQ(εL) strain, indicating that the strengthened ε-clamp interaction is responsible for the increased sensitivity. This increased sensitivity of the dnaQ(εL) strain is due to defective Pol IV-mediated TLS because dnaQ(εL) was epistatic to ΔdinB (SI Appendix, Fig. S2A). The dnaQ(εL) and dnaE(αεM3) strains grew normally and retained nearly wild-type DNA content (SI Appendix, Fig. S2 B and C). Furthermore, the dnaQ(εL) strain retained similar numbers of replisome foci to the dnaQ- strain both in untreated and NFZ-treated cells (SI Appendix, Fig. S2 D and E). The dnaQ(εL) and dnaE(αεM3) strains also retained nearly wild-type SOS-responses to both NFZ and MMS (SI Appendix, Fig. S2F). Collectively, these results rule out the possibility that the increased sensitivity of the dnaQ(εL) strain results from a general defect in DNA replication or the DNA damage response.

ε Subunit Acts as a Gatekeeper. Because the dnaQ(εL) mutation stimulates proofreading of Pol III core (αδθ) (27, 38), we next addressed whether the εL-containing replisome suppressed TLS through futile cycles of TLS and proofreading, impeding the transition from TLS to processive replication (SI Appendix, Fig. S3A) (39, 40). If the proofreading function of the ε subunit counteracts Pol IV-mediated TLS, abrogating the exonuclease activity of the ε subunit should promote Pol IV-mediated TLS. Indeed, when the catalytic residues of the ε subunit were mutated (εLΔD12A,E14A) (41), Pol IV-mediated TLS was promoted (Fig. 3A), indicating that the proofreading function of the ε subunit

Reactions were quenched 12 min after initiation. (Right) Magnified view of the same gel; —, no Pol IV; +, 156 nM Pol IV; numbers (n), number of passages through a N2-FFdG lesion.
antagonizes Pol IV-mediated TLS over N2-FFdG, likely through futile cycles. In contrast to the effect on TLS, abrogating the exonuclease activity of the e subunit reduces replication on the control template (Fig. 3A), indicating that exonuclease activity facilitates processive replication. Importantly, we found that the dnaQ(e2) mutation still strongly suppressed Pol IV-mediated TLS within the exonuclease-dead dnaQ-containing replisome (αεθ–D132A,E143A), which was comparable to the suppression within the wild-type replisome (αεθ) (Fig. 3A, Inset and SI Appendix, Fig. S3B). This result indicates that strengthening the e–cleft interaction suppresses Pol IV-mediated TLS by inhibiting the interaction of Pol IV with the β2 clamp rather than promoting futile cycles of TLS and proofreading.

Given that these results suggest an exonuclease activity-independent gatekeeping role of the e subunit, we hypothesized that weakening the e–cleft interaction or removing e would make replication more potently inhibited by Pol IV and increase Pol IV-mediated TLS. To weaken the e–cleft interaction, we introduced the dnaQ(eαθ) mutation, which substantially decreases affinity for the clamp (K_D > 2 mM) (26). Indeed, the eαθ-containing replisome (ααθ0) exhibited reduced replicative activity on the control template and replication was more potently inhibited by Pol IV as compared with the wild-type replisome (ααθ) (Fig. 3 B, Left Inset). These results indicate that access of Pol IV to the primer/template (P/T) junction during processive replication is inhibited by the e subunit. Consistent with this gatekeeping role, spontaneous mutagenesis was elevated when the e–cleft interaction was weakened (SI Appendix, Table S1). However, identifying the origin of this increase is difficult because weakening the e–cleft interaction impacts not only the access of TLS polymers but also the proofreading activity of Pol III core (27, 38). On the N2-FFdG-containing template, Pol IV-mediated TLS occurred more efficiently in the eαθ-containing replisome compared with the wild-type replisome (ααθ0) when normalized for replication on the control template (Fig. 3 B, Right Inset). Removal of the e subunit further increased the efficiency and potency of Pol IV-mediated TLS (Fig. 3 B, Right Inset), suggesting that in the absence of the e subunit, Pol IV may frequently bind to the αβ subunits even during processive replication. Similar observations were also made with the N2-CEdG-containing template (SI Appendix, Fig. S3C). These results demonstrate that it is a gatekeeping activity of the e subunit rather than its exonuclease activity that limits the access of Pol IV and potentially other clamp-binding proteins to lesion-stalled replisomes.

**Strength of the e–Cleft Interaction Determines Pathway Choice between TLS at the Fork and Repriming.** Given prior observations that the reconstituted *E. coli* replisome can repriming leading-strand synthesis (42, 43), we next asked if suppression of TLS at the fork by weakening the e–cleft interaction increased repriming. To determine whether the N2-FFdG–stalled replisome reprimed downstream of the lesion, we employed Southern blotting to detect the expected repriming products (Fig. 4A and SI Appendix, Fig. S1F). In the absence of Pol IV, replication of the N2-FFdG–containing template created replication products that were detected with leading-strand-specific Southern blot probes (Fig. 4B)
Fig. 3. ε Subunit suppresses TLS through a gatekeeping role. (A) Strengthening the ε-cleft interaction with the dnaQεδ mutation suppresses TLS at the fork for both wild-type and catalytically defective ε subunits. (Upper) RL leading-strand replication products. (Lower) Relative band intensities of these leading-strand replication products; εCD, catalytically defective (εD12A,E144A); εεεε, εCD with the dnaQεδ mutation. (Inset, Right) Replication-normalized RT (relative TLS) at [Pol IV] = 78 nM (mean ± SD, n = 2). (B) Weakening the ε-cleft interaction promotes Pol IV-mediated TLS at the fork. RL leading-strand replication products resulting from replication of a lesion-free control template (Upper, Left) or a N²-FFdG-containing template (Upper, Right) by the indicated Pol III complexes. (Lower) Relative band intensities of these leading-strand replication products. (Inset, Left) Inhibition of replication by Pol IV; replication is normalized to replication in the absence of Pol IV (NR, normalized replication). (Inset, Right) Replication-normalized RT at [Pol IV] = 156 nM (mean ± SD, n > 2); αεθ, wild-type replisome; αεθε, εδ-containing replisome; αεθεθ, ε-free replisome.

and SI Appendix, Fig. S4 A–C) (2). Replication products that ran as discrete bands above the template were leading-strand replication products that resulted from processive replication because these were detected only with leading-strand probes and were longer than the template (Fig. 4 B and SI Appendix, Fig. S4 C). Additionally, we detected diffuse replication products that ran below the template. These replication products were not created in the absence of DnaG, but unlike Okazaki fragments these were detected with leading-strand probes (Fig. 4 B and SI Appendix, Fig. S4 C and D). These short leading-strand products were better detected with a distal leading-strand probe (1,901-nt probe) than a proximal leading-strand probe (40-nt probe) (Fig. 4 A and SI Appendix, Fig. S4 E), consistent with prior reports that repriming occurs a few hundred nucleotides downstream of the lesion (42). Collectively, these results indicate that replisomes stalled at N²-FFdG reprime DNA synthesis downstream of N²-FFdG, leaving an ssDNA gap between the lesion and a newly synthesized leading-strand RNA primer (Fig. 4 A).

The addition of increasing concentrations of Pol IV to replication reactions of the N²-FFdG-containing template led to a gradual increase in TLS at the fork and a concomitant decrease in repriming, suggesting that Pol IV-mediated TLS competes with repriming (Fig. 4 B and SI Appendix, Fig. S4 F–H). Indeed, when wild-type Pol IV (100 nM) was added with varying time delays after the initiation of replication, stalled replisomes were rapidly released into the TLS pathway and no further increase in repriming was observed (Fig. 4 C).

Notably, for the εδ-containing replisome, while TLS was suppressed, repriming persisted in the presence of higher concentrations of Pol IV as compared with the wild-type replisome (Fig. 4 B). To examine whether strengthening the ε-cleft interaction increases the intrinsic propensity of the replisome to reprime, we compared the time course of repriming in the absence of Pol IV between the wild-type and εδ-containing replisomes (SI Appendix, Fig. S4 D). Both replisomes had a similar partitioning between Pol III-mediated TLS over the lesion and repriming with repriming occurring at similar rates (t1/2,apparent ~4 min). Therefore, the persistent repriming of the εδ-containing replisome at higher concentrations of Pol IV most likely results from suppression of Pol IV-mediated TLS.

In Vivo, Pol IV-Mediated TLS Increases upon Weakening the ε–β2 Clamp Interaction. Our in vitro observations demonstrate that the ε-cleft interaction plays a decisive role in determining the fate of lesion-stalled replisomes. To test whether this also happened in living cells, we employed an in vivo assay that quantitatively measures the fraction of stalled replisomes that are resolved either by TLS or via the DA pathway at a site-specific lesion located in the chromosome. The experimental system (SI Appendix, Fig. S5 A and B), which is based on phage-λ site-specific recombination, consists of 2 major components: A recipient E. coli strain with a single attR site, and a nonreplicating plasmid construct containing the single lesion of interest, an attL site, and an ampicillin resistance gene. A site-specific recombination reaction between attL and attR is controlled by ectopic expression of phage-λ integrase and excisionase proteins (44) and leads to the integration of the lesion-containing plasmid into the chromosome. Integrants are selected based on their resistance to ampicillin. The chromosomal region where integration takes place carries the 3′-end of the lacZ gene fused to attR (minute 17 in the E. coli chromosome), while the remaining 5′-end is located on the incoming plasmid fragment in fusion with attL. Precise integration restores a functional β-galactosidase gene (lacZ*). A short sequence heterology inactivates the lacZ gene in the “opposite” strand across from the single lesion and serves as a genetic marker to allow strand discrimination during replication (45). The recipient strain carries both nvrA and mutS deletions to prevent the single lesion and the sequence heterology from being repaired by nucleotide excision repair and mismatch repair, respectively. Functional LacZ is expressed only when the lesion-containing leading-strand template is bypassed by TLS events with the exception of events in which a frameshift mutation is introduced (SI Appendix, Fig. S5 B and C). Therefore, colonies resulting from TLS events appear as blue/white-colored colonies on X-Gal–containing plates (46). On the other hand, white colonies result from RecA-mediated HDR (47). For most blue/white-colored colonies observed, the blue and white sectors were
To investigate Pol IV-mediated TLS, we introduced a single (−)-trans-anti-benzopyrene-N²-dG adduct [dG-BaP(−)] into the E. coli genome, known to be bypassed only by Pol IV (48, 49). In the dnaQ⁺ background, the majority of G-BaP(−)-stalled replisomes were resolved by the DA pathway, while only 20% by TLS (Fig. S4). However, in the dnaQ(ε0⁺) background, which weakens the ε-cleft interaction, 55% of the stalled replisomes were resolved by TLS with a corresponding drop in the use of the DA pathway. As the basal SOS response in the dnaQ(ε0⁺) strain was comparable to that in the dnaQ⁺ strain (SI Appendix, Fig. SSD), we ruled out the possibility that the increase in Pol IV-mediated TLS in the dnaQ(ε0⁺) strain resulted from elevation of Pol IV expression levels.

As the dnaQ(ε0⁺) mutation also reduces the processivity of proofreading (27, 38), we examined if the increase in TLS in dnaQ(ε0⁺) background was primarily due to a decrease in proofreading activity. To test this possibility, we abolished the exonuclease activity of the ε subunit with the mutD5 mutation (SI Appendix, Table S1A) (50, 51) and assessed the fraction of DA and TLS events over the G-BP(−) lesion. In the mutD5 background, participation of TLS increased from ~20 to ~34% but still remained significantly below the 55% value seen in the dnaQ(ε0⁺) strain (Fig. S4). Therefore, the additional increase in TLS seen upon weakening the ε-cleft contact supports the model that the ε subunit plays a gatekeeping role in regulating access of Pol IV during TLS in living cells.

The ε Subunit Acts as a Gatekeeper for all 3 E. coli TLS Polymerases.

Next, we asked whether the ε-cleft interaction plays a similar gatekeeping role in Pol V-mediated TLS across TT-cyclobutane pyrimidine dimer (CPD) and TT(6–4) lesions. In the dnaQ⁺ background, replisomes stalled at TT-CPD and TT(6–4) were fully processed by the DA pathway as no Pol V-mediated TLS was measured. This lack of TLS is likely due to the absence of the active form of Pol V (UmuDεC) as UmuD is not processed into UmuD⁺ under non-SOS-induced conditions (Fig. 5B) (52). To observe Pol V-mediated TLS, we engineered a strain that carries umuD⁺ instead of umuD at the native umuDC locus (umuDC strain); this strain constitutively expresses the active form of Pol V (SI Appendix, Table S1B) (53). In this background, Pol V-mediated TLS corresponded to ~20% and ~5% for TT-CPD and TT(6–4) lesions, respectively (Fig. 5B). Introduction of the dnaQ(ε0⁺) allele in the umuDC background further stimulated TLS to ~70 and ~50% for TT-CPD and TT(6–4), respectively (Fig. 5B). In contrast, introducing the proofreading-deficient mutD5 allele in the umuDC background, had no effect on the level of TLS, suggesting that the robust increase in TLS in the dnaQ(ε0⁺) strain resulted from an increased accessibility of Pol V to the clamp rather than from a defect in proofreading (Fig. 5B).

Finally, to investigate Pol II-mediated TLS, we introduced an N²-acetylaminofluorene dG (G-AAF) lesion in the NarI sequence context (SI Appendix, Supplemental Methods); this lesion can be bypassed by either Pol II- or Pol V-mediated TLS leading to ~2 or error-free bypass, respectively (Fig. 5C) (5, 54). In contrast to what was observed for Pol IV and Pol V cognate lesions, Pol II-mediated TLS over the G-AAF lesion was comparably stimulated in the dnaQ(ε0⁺) and mutD5 strains (Fig. 5D). The similar levels of TLS in the dnaQ(ε0⁺) and mutD5 backgrounds are most likely related to the peculiar mechanism of Pol II-mediated ~2

Fig. 4A. (Right) Quantitation of repriming products. Amounts of repriming products were plotted relative to the amount of repriming products detected at the final time point (720 s) in the absence of Pol IV. The lines are linear connections of immediate time points (mean ± SD, n = 3).
frameshift mutagenesis, which involves elongation of a “slipped lesion terminus” (Fig. 5C) (54, 55). As revealed in the crystal structure, Pol II is uniquely suited to elongate this frameshift intermediate (56). Upon stalling at the G-AAF lesion, Pol III dissociates from the P/T junction, allowing for the formation of a −2 frameshift intermediate; this intermediate is thermodynamically favored by the stacking energy provided by insertion of the aromatic fluorene residue between the neighboring base pairs (57, 58). We speculate that Pol II-mediated TLS was promoted in both backgrounds because in the mutD5 strain the formation of the −2 slipped intermediate is facilitated due to the lack of Pol III-dependent proofreading, while in the dnaQεεεεε strain, Pol II has increased access to the clamp, resulting in promoted extension of this slipped intermediate.

With respect to error-free bypass of G-AAF by Pol V, TLS is stimulated (−7-fold) in the dnaQεεεεε strain compared with the mutD5 strain (Fig. SD), consistent with the effect of the dnaQεεεεε on Pol V-mediated TLS across TT-CPD and TT(6-4) lesions. As weakening the ε-cleft interaction in cells significantly increased the utilization of TLS over cognate lesions for all 3 TLS polymerases, we conclude that the ε-cleft contact acts as a gatekeeper to regulate access to the P/T junction.

Discussion

Conformational Transitions of the Replicative Polymerase Underlie Pathway Choice. During replication, replisomes encounter a diverse set of challenges that can stall replicative DNA polymerases, including DNA lesions and protein-DNA complexes (59, 60). It remains poorly understood how replisomes select among distinct rescue mechanisms to cope with these different situations. In this study, we demonstrated that for replisomes stalled at leading-strand lesions, the dynamic interaction between the exo subunit (ε) of Pol III and the βclamp plays a crucial role in pathway choice between TLS at the fork and repriming (Fig. 6A). We showed that the ε-cleft interaction acts as a steric gate to limit access of TLS polymerases to the P/T junction in a manner that is largely independent of its exonuclease activity. This model is consistent with in vivo imaging demonstrating that Pol IV is only weakly colocalized with the replisome in undamaged cells (14).

Upon polyclamping at a lesion, Pol IV gains transient access to the P/T junction by first binding to the cleft on the βclamp, resulting in promoted extension of this slippage intermediate. We showed that the ε-cleft interaction increases utilization of TLS in cells (Fig. 5A) (54, 55), consistent with the effect of the dnaQεεεεε on Pol V-mediated TLS across TT-CPD and TT(6-4) lesions. As weakening the ε-cleft interaction in cells significantly increased the utilization of TLS over cognate lesions for all 3 TLS polymerases, we conclude that the ε-cleft contact acts as a gatekeeper to regulate access to the P/T junction.
Within stalled replisomes, recurring binding and unbinding between the ε subunit and the αε-cleft form a kinetic barrier to Pol IV-clamp binding. Unbinding may be stimulated by a bulky lesion in the template strand; the inability of the narrow catalytic cleft of the replicative polymerase to accommodate the lesion-containing template could promote opening of the Pol III-clamp complex. Alternatively, replicating Pol III core complexes may sample the open state (reversible transition between b and c in Fig. 6d) regardless of the presence of blocking lesions (61). In either case, strengthening the ε–cleft interaction suppresses these conformational transitions and therefore TLS, while weakening this interaction has the opposite effect.

Recent observations demonstrate that the E. coli replisome readily exchanges Pol III holoenzyme from cytosol during processive replication (62–64), suggesting that Pol III core dissociation from the clamp may be required for Pol IV binding (61). In contrast, our results suggest that the Pol III core remains bound to the β2 clamp during Pol IV-mediated TLS as strengthening the α–cleft interaction had little effect on TLS in vitro and in cells. This model is consistent with prior observations that both Pol III core and Pol IV can bind the clamp simultaneously, the toolbelt model (9, 65, 66). Single-molecule imaging of a minimal reconstitution of β2 clamp, Pol III core, and Pol IV demonstrated that higher concentrations of the Pol III core and Pol IV promoted simultaneous binding of the clamp (61); this simultaneous binding could be facilitated by interactions between polymerases and DNA that locally concentrate Pol III core and Pol IV. We propose that during exchange of the Pol III holoenzyme, another Pol III core rapidly binds the β2 clamp due to the preassociation of the Pol III holoenzyme with the replisome through its interaction with the DnaB helicase (67, 68) (SI Appendix, Fig. S6d). In essence this is a “dynamic toolbelt” model, in which rapid exchange of the Pol III core happens through the α–cleft while Pol IV carries out synthesis. Within this model the α–cleft interaction is maintained, which is essential to prevent other factors from accessing the P/T junction and compromising processive replication (7, 24).

It is also likely that the ε–cleft interaction limits the size of the TLS patch. Pol IV is expected to synthesize only a short patch before it falls off the template and loses its CBM–cleft interaction (transitions from c through f in Fig. 6d) (9, 37). Following the dissociation of Pol IV from the ε–cleft, the Pol III core must reestablish its ε–cleft interaction before it can resume processive replication (transition from a to b in Fig. 6d). This step would be much faster than expected for diffusion-controlled binding of the Pol III core if Pol III remained bound to the β2 clamp while Pol IV performs TLS. Conversely, weakening the ε–cleft interaction could result in longer TLS patches and slower replication due to rebinding of Pol IV (Fig. 6d, transition from f to d rather than to a).

Repriming Is a Failsafe Mechanism for Rescuing Stalled Replisomes.

We demonstrated that TLS and repriming compete kinetically to resolve stalled replisomes. Failure to carry out TLS at the fork in a timely manner results in repriming, which acts as a failsafe to ensure replisome progression. A consequence of repriming is the formation of an ssDNA gap behind the fork (SI Appendix, Fig. S6b). This gap is initially coated by an ssDNA binding protein and subsequently converted to an RecA–ssDNA filament, which induces the SOS DNA damage response. Alternatively, TLS at the fork yields a continuous DNA product and therefore does not lead to induction of the SOS response.

Within our reconstitution, repriming downstream of an N2–FFdG adduct on the leading-strand template occurred quite slowly compared with Pol IV-mediated TLS at the fork. Similar to prior observations of repriming downstream of CPD lesions, we observed repriming occurring on the timescale of ∼4 min, implying that the rate of repriming is likely independent of lesion identity (42, 43). While direct measurements of the rate of repriming in cells have not been made, estimates (~10 s) suggest that it could be much

processive replication. Strengthening the ε–cleft interaction ("closing the gate," transition from c to b in Fig. 6d) exerted the opposite effects. Therefore, the strength of this interaction is finely tuned to maximize processive replication on undamaged DNA while still enabling replisome remodeling by TLS polymerases upon the encounter of DNA damage.

![Fig. 6](https://example.com/fig6.png)

**Fig. 6.** Conformational basis of pathway choice of lesion-stalled replisomes. (A) Conformational transitions of Pol III core complex during TLS. (a) Pol III core (αɛ) occupies both ε- and ε-clefts during processive replication. (b and c) Upon encountering a lesion on the leading strand, Pol III core swings away from the P/T junction while remaining bound to the β2 clamp through the α–cleft interaction. This conformational transition is either facilitated or suppressed by the dnaQεQ or the dnaQεQ mutations, respectively. (d and e) Pol IV binds to the ε–cleft and take over the P/T junction and performs TLS past the lesion. (f) After synthesizing a short patch, Pol IV swings away from the P/T junction and Pol III core reestablishes the ε–cleft interaction and resumes processive replication. Within closed states, states a and b, the ε–cleft is occupied by the ε subunit. Within open states, states c though f, the ε–cleft is not occupied by the ε subunit. The β subunit of Pol III core is not depicted for simplicity. (B) Pathway choice for lesion-stalled replisomes during the SOS response: Repriming followed by gap-filling TLS vs. TLS at the fork.
faster than in vitro observations (69, 70). Therefore, it is possible that other factors present in the cellular environment, but not in current biochemical reconstitutions, may facilitate repriming.

On the other hand, the rate of TLS is controlled by at least 2 factors: 1) the rate at which a TLS polymerase can bind the P/T junction and 2) the rate at which the TLS polymerase can insert and extend past the lesion. As we have observed here, access of a TLS polymerase to the P/T junction at the replication fork is regulated, in part, by the dynamics of the ε gate. Increasing concentrations of Pol IV led to more frequent TLS at the fork within our biochemical reconstitution. Similarly, when the level of Pol IV was constitutively elevated as a result of eliminating LexA repressor binding sites in the promoter of the dinB gene (dinBp-lexA-mut), the MMS-induced SOS response was decreased relative to the wild-type strain, indicating that creation of ssDNA gaps is suppressed (Fig. 6B and SI Appendix, Fig. S6 B and C). These results show that higher levels of Pol IV help to overcome the gatekeeping barrier of the ε subunit by enabling Pol IV to more readily capture the ε open state.

As cellular concentrations of all 3 TLS DNA polymerases are increased upon induction of the DNA damage-dependent SOS response, it is likely that TLS at the fork plays a more prominent role in rescuing lesion stalled replisomes after SOS induction. During the early stages of the SOS response, when levels of TLS polymerases are relatively low, the majority of lesion-stalled replisomes are resolved by lesion skipping and most of the resulting ssDNA gaps are repaired by DA (Fig. 6B). This is supported by our in vivo observations that only small fractions of lesion-stalled resolvases were resolved by TLS, which is likely through a gap-filling process (Fig. 5 A, B, and D). A potential exception is Pol IV, which is present at relatively high levels even in the SOS uninduced state and can efficiently bypass cognate lesions (SI Appendix, Fig. S1I). In a ΔdinB strain, resolvases stalled at Pol IV cognate DNA lesions would be predominantly resolved by lesion skipping, leaving more ssDNA gaps than in a dinB strain. Indeed, when Pol IV-mediated TLS was completely abolished by the deletion of dinB, the MMS-induced SOS response was highly elevated compared with the wild-type strain (SI Appendix, Fig. S6C), consistent with an increase in repriming. Collectively, pathway choice is a net result of 2 counteracting molecular events—1) gatekeeping by the ε subunit and 2) mass action of TLS polymerases (71)—with the latter changing throughout the SOS response. Failure of the TLS polymerase to gain access to the P/T junction or the inability to quickly bypass the lesion upon binding results in repriming and subsequent gap repair.

The Competition between TLS at the Fork and Repriming, and Its Impact on Mutagenesis. TLS at the fork prevents the creation of ssDNA gaps by outcompeting repriming. This gap-suppressing effect of TLS at the fork may play a pivotal role in determining the extent of damage-induced mutagenesis. A recent study has demonstrated that damage-induced mutagenesis is not limited to the lesion site but instead can spread a few hundred nucleotides from the lesion (72). This extended patch of low-fidelity synthesis likely results from gap filling synthesis where bypass of the lesion by the cognate TLS polymerase is followed by the sequential action of multiple polymerases, including the highly mutagenic Pol V, to fill in the remaining ssDNA gap (Fig. 6B) (73, 74). However, if TLS occurs at the fork, error-prone TLS may be limited to around the blocking lesion, as Pol III can readily regain control of the P/T junction (Fig. 6B).

Within cells the extent to which TLS occurs at the fork versus in an ssDNA gap created by repriming remains unclear. Lesion identity, which plays a role in determining the rate of TLS, almost certainly affects the partitioning between these pathways. Lesions that are strongly blocking, such as UV-induced lesions, are likely to be predominantly resolved through repriming due to the absence of Pol V in the early stage of the UV-induced SOS response (75). Even in the presence of highly elevated levels of Pol V, gap filling may still be the predominant mechanism as Pol V activation requires a RecA-ssDNA filament downstream of the stalled replisome (76–78). Weakening the ε-gate still dramatically increased Pol V-mediated TLS in cells (Fig. 5B), suggesting that the interaction between Pol III and the β2 clamp likely influences polymerase switching independent of whether TLS occurs at the fork or in a gap (Fig. 6B). Biochemical studies presented here and in prior work, demonstrate that Pol IV can efficiently carry out TLS at the fork for at least a subset of lesions (79, 80). Cellular evidence for Pol IV-mediated TLS at the fork remains limited but the role of dinB in MMS-induced mutagenesis provides circumstantial evidence. 3meA is the major replication-blocking lesion created in MMS-treated cells and is bypassed primarily by Pol IV in a largely error-free manner (81–84). Intriguingly, deletion of dinB increases MMS-induced mutagenesis, which may result from more mutagenic TLS over 3meA by other polymerases at the fork (84, 85). However, we speculate that this increase is likely related to the creation of ssDNA gaps due to frequent repriming. In the absence of Pol IV, other polymerases mediate TLS over methyl lesions primarily in a postreplicative manner because inefficient synthesis past the lesion is likely outcompeted by repriming (Figs. 4B and 6B). As is the case for untargeted mutagenesis by UV lesions (72), the remainder of the gap can be filled in by mutagenic gap-filling synthesis with Pol V being the major mutator. In contrast, MMS-induced mutagenesis is reduced by constitutive expression of Pol IV (85). These observations are consistent with Pol IV-mediated TLS at the fork and support work on ssDNA gaps (Fig. 6B).

Our result that the ε-gate antagonizes TLS raises the question if other factors may stimulate TLS in cells and therefore contribute to mutagenesis. Intriguingly, we previously showed that Pol IV is highly enriched near replisomes upon DNA damage in a manner that was only partially dependent on interactions with the β2 clamp (14), suggesting that additional interactions, possibly with replisome components, are required for localization of Pol IV to stalled replisomes. Identifying these putative Pol IV–replisome interactions, along with factors that may promote repriming, will be important areas of future investigation.
**In Vivo Tolerance Assay and Sensitivity Assay.** To assess partitioning of stalled replications into either DA or TLS, a single replication blocking lesion was incorporated into the E. coli genome, as previously described (46). Subsequently lesion-incorporated cells were grown on X-gal-containing LB agar plates, and the next day white and blue/white-colored colonies, representing DA and TLS events, respectively, were counted. These colony counts were used to calculate fraction of cell death described in Supplemental Methods. To examine sensitivity of strains to DNA-damaging agents, overnight cultures were serially diluted and spotted onto NZ- or MMS-containing LB-agar plates and incubated overnight. Detection spots were pictured and analyzed.

All data are included in the main text and the SI Appendix. More details on materials and methods can be found in the SI Appendix.

**ACKNOWLEDGMENTS.** We thank Shingo Fujii (CNRS, Marseille), Daniel Semlow (Harvard Medical School), Johannes Walter (Harvard Medical School), and members of the J.J.L. laboratory for helpful discussions and comments on the manuscript; and Deyu Li (University of Rhode Island) and Yinsheng Wang (University of California, Riverside) for providing the N2-FdG– and N2-CEdG-containing oligomers, respectively. This work was supported by the National Institutes of Health Grants R01 GM114065 (to J.J.L.) and F32 GM113516 (to E.S.T.); Agence Nationale de la Recherche FReR Project ANR 11 BSVH 01 07 (to R.P.F.); and the Australian Research Council DP150100956 and DP180100858 (to N.E.D.).
