ARTICLES

Preferential D-loop extension by a translesion DNA polymerase underlies error-prone recombination

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Although homologous recombination is considered an accurate form of DNA repair, genetics suggest that the Escherichia coli translesion DNA polymerase IV (Pol IV, also known as DinB) promotes error-prone recombination during stress, which allows cells to overcome adverse conditions. However, how Pol IV functions and is regulated during recombination under stress is unknown. We show that Pol IV is highly proficient in error-prone recombination and is preferentially recruited to displacement loops (D loops) at stress-induced concentrations in vitro. We also found that high-fidelity Pol II switches to exonuclease mode at D loops, which is stimulated by topological stress and reduced deoxyribonucleotide pool concentration during stationary phase. The exonuclease activity of Pol II enables it to compete with Pol IV, which probably suppresses error-prone recombination. These findings indicate that preferential D-loop extension by Pol IV facilitates error-prone recombination and explain how Pol II reduces such errors in vivo.

Homologous recombination repairs double-strand breaks (DSBs) by directing replication to copy sequence information from a homologous donor (Fig. 1a)1–4. For example, after formation of a DSB, nuclease resect the DNA, resulting in a 3′ single-stranded DNA (ssDNA) tail. RecA-type recombinases form a filament along the tail that facilitates strand invasion within a homologous donor DNA, resulting in a D loop. DNA polymerase then extends the 3′ end of the invading strand using the complementary strand within the donor DNA as a template, a process called recombination-directed replication (RDR) or D-loop extension. Recombination intermediates are then further processed to form Holliday junctions, which are resolved by endonucleases.

The accuracy of homologous recombination, which is widely considered to be high, is dependent on the fidelity of RDR. Although high-fidelity replicative polymerases predominantly perform RDR5–8, mounting evidence indicates that low-fidelity translesion polymerases also carry out this function, suggesting that homologous recombination is error prone. For example, previous studies in eukaryotes, including humans, yeast, chickens, frogs and flies, have indicated that low-fidelity translesion polymerases η, v, θ and ζ contribute to RDR, which probably promotes mutagenesis9–15. Moreover, recent evidence from Drosophila melanogaster suggests that translesion polymerases compete with replicative polymerases during homologous recombination16. Although translesion polymerases are widely known to promote replication past lesions in DNA, it is becoming clear that these low-fidelity enzymes function during homologous recombination in various organisms and therefore may have been selected to perform RDR in all domains of life.

In E. coli, several years of genetic studies have suggested that the Y-family translesion Pol IV (DinB) promotes error-prone homologous recombination in the form of mutations during stress specifically16–20. Such error-prone recombination allows E. coli to rapidly evolve and overcome stressful conditions, including nutritional starvation and exposure to antibiotics16,19. Pol IV–induced recombination errors are therefore also referred to as stress-induced or adaptive mutations. Our current knowledge of Pol IV involvement in error-prone homologous recombination is based mostly on genetic data. For example, previous genetic studies have demonstrated that Pol IV–induced mutations are targeted to regions of DSBs and require SOS-induced levels of Pol IV (~2,500 molecules per cell), recombination factors (RecA, RecBCD and RuvABC) and the RpoS stress response, which further upregulates Pol IV (~100%) and downregulates mismatch repair16,17,19,21,22.

Current models based on these findings propose that Pol IV promotes mutations near D loops during homologous recombination under stress17,20. Yet clear evidence for Pol IV RDR activity, which is outside its normal role in translesion synthesis, has never been demonstrated. Furthermore, how Pol IV is recruited, regulated and competes with other polymerases during homologous recombination under stress remains unclear. Here we sought to provide mechanistic insight into the activity and regulation of Pol IV during homologous recombination under stress.

RESULTS

Pol IV is proficient and error prone in RDR

We used a biochemical approach to investigate the activity and regulation of Pol IV in RDR (D-loop extension) (Fig. 1b). The results demonstrated

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that Pol IV promotes RDR (Fig. 1c, lanes 1–4), which requires Pol IV and RecA (Fig. 1d). In contrast, the related Y-family Pol V (UmuD’/C) did not perform RDR under identical conditions (Fig. 1c, lanes 5–8). The inability of Pol V to promote RDR was surprising to us, as its activity requires RecA filaments in trans, which are present in excess, as indicated by the free ssDNA (Fig. 1c, lower band)\textsuperscript{23}. Nevertheless, we repeated the reaction with Pol V but added more than twice the amount of RecA along with increasing concentrations of unlabeled heterologous trans ssDNA, which can not form a D loop. Pol V still did not promote RDR (Fig. 1e). As a positive control, we demonstrated that the same amount of Pol V extends a simple primer template that requires ssDNA and RecA in trans, as shown previously (Fig. 1f)\textsuperscript{23}. Considering that Pol V has a small role in promoting stress-induced mutations during recombination, additional factors may be needed to stimulate its activity at D loops\textsuperscript{24}.

**Figure 1** Pol IV is highly proficient and error prone in recombination-directed replication. (a) Model of DSB repair. DNA ends are resected by nucleases, resulting in 3’ ssDNA tails. RecA promotes strand invasion, resulting in a D loop. Pol extends the D loop (red arrow). The second DNA end is captured, and Holliday junctions are formed, which are subsequently resolved by an endonuclease. (b) Scheme for reconstitution of RDR (D-loop extension). A 5’,32P-labeled ssDNA is incubated with RecA, ATP and dNTPs, promoting RecA filament formation. A supercoiled plasmid containing the same sequence as the ssDNA is then added, facilitating D-loop formation. The β-clamp, which confers processivity onto polymerases, is then assembled at the D loop by adding β along with its clamp loader (the γ complex) and ssDNA binding protein (SSB). DNA polymerase is then added, initiating RDR by extending the D loop, nt, nucleotide. (c) Blots are for western, northern or southern gels using antibodies. Throughout paper, all gels visualizing radiolabeled DNA products used no antibodies. Gel showing RDR performed with 500 nM Pol IV (lanes 1–4) or Pol V (lanes 5–8) for the indicated times. (d) Gel of the controls for Pol IV RDR activity performed as in c in the presence or absence of the indicated reagents. (e) Gel showing RDR performed with 500 nM Pol V in the presence of increasing amounts of ssDNA and 3.3 µM RecA. (f) Gel showing primer extension performed with 500 nM Pol V and 2 µM RecA in the presence (lane 3) or absence (lane 2) of 160 nM trans ssDNA. The asterisk indicates 32P. (g) Gels showing RDR performed with Pol IV at relative concentrations corresponding to those in SOS-induced cells in the presence (lane 3, left gel) and absence (lane 2, left gel, and the entire right gel) of β with (right gel) or without (left gel) increasing amounts of sodium glutamate (NaGlu). RE, relative extension. (h) Gels of primer (left) and D-loop (right) extension performed with Pol IV and the indicated dNTP. (i) Gels of D-loop extension performed as in h. (j) Gels showing RDR performed with Pol IV in the presence of 50 µM deoxyguanosine triphosphate (dGTP) and 10 µM 2’,3’-dideoxyadenosine triphosphate (ddATP) (lane 1). Incorporation of the 2’,3’-dideoxynucleoside monophosphate (dAMP) chain terminator opposite the thymidine base prevents further extension of the D loop. DNA products were analyzed as in h. The DNA sequence of the product in lane 1 was determined by comparison to the DNA markers in lanes 2 and 3. The mobility of the product in lane 1 (upper band) corresponds to the marker in lane 1, indicating that the D loop was extended by the incorporation of three dGMPs and one ddAMP demonstrating a −1 frameshift mutation (see schematic at bottom). Partial DNA sequences of the invading ssDNA and markers were present in all reactions except where indicated.
We next examined whether the replication cofactor β, which confers processivity onto polymerases, is required for Pol IV RDR activity. We used a relatively high ratio of polymerase to D loop that we based on approximate conditions in SOS-induced cells (Table 1). For example, Pol IV is highly upregulated (~10^4) by the SOS response to ~2,500 molecules per cell, making it the most abundant DNA polymerase in stressed cells (Table 1). Because the average number of DSBs leading to D loops in stressed cells is unknown, it is difficult to model in vitro. We therefore used an approximation of four DSBs per cell, as previous estimates have suggested that each chromosome incurs a break, and E. coli contains four chromosomes in rich medium. Because DSBs result in two DNA ends capable of forming D loops, this results in a polymerase-to–DNA end ratio of 312.5:1 for Pol IV (Table 1). Considering that SOS-induced cells may contain fewer than four DSBs, this ratio may under represent the amount of Pol IV relative to D loops. Nevertheless, using these relative amounts, which take into account the approximate concentration of D loops formed in our assay (Supplementary Fig. 1), we showed that Pol IV is able to promote RDR in the absence of β (Fig. 1g, left), even under conditions of high ionic strength (Fig. 1g, right). This result was unexpected to us and is in contrast to previous biochemical studies of yeast proteins that have demonstrated that proliferating-cell nuclear antigen (PCNA), the eukaryotic equivalent of β, is required for RDR by replicative Pol δ and translesion Pol η. Our results suggest that Pol IV may not require β for its involvement in RDR because of its abundance in stressed cells.

We next investigated the ability of Pol IV to promote mutations at D loops, which is thought to be the central mechanism of error-prone recombination. To our knowledge, the fidelity of polymerases on D loops has not previously been investigated in vitro. As a control, we first examined the fidelity of Pol IV on a primer template that resembles the DNA substrate used during translesion synthesis. The results showed that Pol IV strongly discriminates against incorporating incorrect nucleotides on a primer template (Fig. 1h, left). In contrast, Pol IV showed a high efficiency of nucleotide misincorporation on a D loop under similar conditions and sequence contexts, as in the previous experiment with the primer template (Fig. 1h, compare right and left gels). Pol IV also seems to be more prone to extension on the D loop (Fig. 1h, right). These data suggest that RDR may be inherently error prone. Alternatively, as RecA interacts with Pol IV and collaborates with UmuD to modulate the fidelity of the polymerase, the recombinase may reduce the accuracy of Pol IV on the D loop.

Considering that genetic data strongly implicate Pol IV in promoting mutations during DSB repair under stress, our observation of Pol IV mutagenic activity on a D loop provides the molecular basis for its involvement in stress-induced mutagenesis. A comparison of Pol IV activity on D loops with different sequences suggested that the polymerase preferentially misincorporates the nucleotide that is complementary to the +2 template base, which is located two positions downstream from the 3′ end of the invading ssDNA (compare Fig. 1h, right and Fig. 1i). For example, Pol IV preferentially misincorporated deoxycytidine monophosphate (dCMP), which is complementary to the +2 template base guanosine (Fig. 1h, right). In contrast, deoxyguanosine monophosphate (dGMP) is preferentially misincorporated when cytosine is the +2 template base (Fig. 1i). Previous biochemical studies have shown that Pol IV is able to ‘skip over’ the correct template base, facilitating a −1 frameshift mutation. The ability of Pol IV to promote −1 frameshifts has been widely used to detect its activity in error-prone recombination. Consistent with these previous studies, we showed that Pol IV is capable of promoting a −1 frameshift on a D loop (Fig. 1j). Notably, Pol IV–induced mutations probably go unrepaired during stationary phase, when mismatch repair is deficient. These data demonstrate that Pol IV is highly proficient in error-prone RDR, providing mechanistic insight into its role in stress-induced mutagenesis.

Mechanism of Pol IV recruitment to D loops

Given that the replisome performs RDR in unstressed proliferating cells, an important consideration is how Pol IV gains access to D loops during stress. As SOS concentrations of Pol IV are required for its role in error-prone recombination, we reasoned that upregulation of the polymerase might facilitate its recruitment to D loops. For example, polymerase access to D loops in proliferating cells is prevented by the primosomal protein PriA, which binds tightly to D loops, where it recruits other primosomal proteins to assemble the replisome during RDR (Fig. 2a, left). Consistent with previous studies, PriA inhibited D-loop extension by replicative Pol III (Fig. 2a, right). Similarly, concentrations of PriA corresponding to known amounts in the cell (~70 molecules per cell) suppressed RDR by Pol IV at levels corresponding to those in nonstressed cells (~250 molecules per cell).

Table 1 Relative amounts of translesion polymerases in SOS-induced cells

| Polymerase | +SOS mol per cell | Approximate mol per chromosome | Estimated mol per DNA end |
|------------|------------------|-------------------------------|---------------------------|
| Pol IV     | 2,500            | 625                           | 312.5                     |
| Pol V      | 200              | 50                            | 25                        |
| Pol II     | 350              | 87.5                          | 43.8                      |

+SOS, under SOS conditions; mol, number of molecules.

**Figure 2** High levels of Pol IV comparable to those in SOS-induced cells facilitate its recruitment to D loops. (a) Schematic of PriA inhibition of D-loop extension by Pol III (left) and gel showing RDR performed with Pol III and the indicated concentrations of PriA (right). (b) Gel showing RDR performed with Pol IV concentrations corresponding to non-SOS (left gel) and SOS (right gel) conditions in the presence (lanes 2) and absence (lanes 1) of relative cellular levels of PriA (175 nM). Also shown are models of competition between Pol IV and PriA at D loops during nonstressed and stressed conditions (right). RE, relative extension. β-clamp, clamp loader and SSB were present in all reactions.
Figure 3  Pol II switches to an active exonuclease mode at D loops. (a) A time course of RDR performed with WT (left) or exonuclease-deficient (exo−; right) Pol II. (b) A time course of replication performed by WT and exonuclease-deficient Pol II on a linear dsDNA template (left) or a circular primer-template substrate (right). (c) Gel showing RDR performed with WT Pol II in the presence (lane 2) or absence (lane 1) of gyrase. (d) Model of Pol II activity at D loops.

preferential RDR activity by Pol IV in the presence of RuvAB, which also binds tightly to D loops and promotes stress-induced mutations (Supplementary Fig. 2). We further found that levels of Pol IV comparable to those in SOS-induced cells promote RDR in the presence of all the primosomal and replisome components and supersede
We next investigated whether Pol II performs RDR (Fig. 3). We found that although Pol II initially extends the D loop, the reaction is subsequently reversed, presumably because of the enzyme’s $3'\rightarrow5'$ exonuclease activity (Fig. 3a, left). Indeed, the reverse reaction was not performed by a previously characterized mutant version of Pol II (D155A E157A) that is deficient in exonuclease activity (Fig. 3a, right)\(^37\). Such constant exonuclease activity by a DNA polymerase is unprecedented considering that the reaction was performed with a saturating deoxyribonucleotide (dNTP) concentration (50 μM) that exceeds the enzyme’s $K_m$ for dNTPs (3.7 μM)\(^38\). Given that dNTP pools are reduced to similar concentrations (~35–50 μM) during stationary phase\(^39\) and concentrations of Pol II corresponding to SOS conditions gain access to D loops in the presence of PriA (Supplementary Fig. 4), the observed phenomenon is probably relevant to Pol II activity in stressed cells and thus warranted further investigation.

We hypothesized that the exonuclease domain of Pol II is stimulated as a result of the inhibition of forward movement by topological constraint in the DNA generated by positive supercoils during D-loop extension. Consistent with this notion, we demonstrated that wild-type (WT) and exonuclease-deficient Pol II act similarly on a linear double-stranded DNA (dsDNA) template and a circular primer template, indicating that the observed exonuclease activity is specific to D loops (Fig. 3b). Our hypothesis was further supported by the effects of gyrase, which removes positive supercoils in DNA and suppressed the reverse reaction (Fig. 3c); this effect seemed to be due to a slight delay in Pol II exonuclease activity (Supplementary Fig. 5). These results support a model in which superhelical tension generated during D-loop extension promotes the reverse translocation and exonuclease function of Pol II, facilitating a switch to a highly active exonuclease mode (Fig. 3d). This model is supported by biophysical studies demonstrating that mechanical tension on the DNA template stimulates proofreading by bacteriophage phi29 polymerase\(^40\). We further found that reducing the dNTP pool concentration to 10 μM facilitated the reverse reaction (compare Fig. 3e with Fig. 3a, left), whereas increasing the dNTP pool concentration to 100 μM, which reflects the conditions in proliferating cells, either during (Fig. 3f) or before (Fig. 3g) the reaction prevented Pol II from switching to exonuclease mode during the same timecourse\(^39\). These findings suggest that the enzyme’s $K_m$ for dNTPs is increased under conditions of opposing force (superhelical tension).

We next compared RDR by WT and exonuclease-deficient Pol III to determine whether this exonuclease activity is specific to Pol II. We used similar conditions to those with Pol II (50 μM dNTPs); however, we substituted the γ subunit of the clamp loader with a γ subunit, which specifically binds to Pol III and facilitates its recruitment to DNA; γ is the full-length version of γ, which is truncated due to a translational frameshift\(^41\). The results showed that exonuclease-deficient Pol III extends D loops further than WT Pol III (Fig. 3h) but acts the same as WT Pol III on a primer template (Fig. 3i) and within the replisome (Fig. 3j). Hence, the exonuclease domain of Pol III was also activated at D loops. However, only Pol II had the ability to reverse the D-loop extension reaction, which we attribute to its highly active exonuclease domain compared to that of Pol III (Fig. 3k).

Pol II requires exonuclease activity to compete with Pol IV

We next examined competition between Pol II and Pol IV by performing RDR in the presence of both enzymes using their relative concentrations in SOS-induced cells (Table 1). As each polymerase produced a distinct product, we were able to determine which enzyme acts dominantly. The results showed that Pol IV competes with Pol II...
Genetics also implicate Pol IV in RDR during replication restart 43. However, in vitro evidence supporting Pol IV RDR activity has been lacking. This report verifies the ability of Pol IV to promote RDR in a reconstituted assay, which, in conjunction with previous genetics data, establishes a new DNA repair function for this enzyme. Our data further show that Pol IV is efficient in misincorporation and mismatch extension during D-loop extension, consistent with its ability to generate mutations during homologous recombination in stressed cells. The high proficiency of Pol IV in error-prone RDR and its abundance in growth-limited cells (~5,000 molecules per cell)27,20 provide an explanation of why this enzyme is responsible for the majority of (85%) stress-induced mutations 18.

Notably, we found that Pol IV is considerably more error prone on a D loop compared to a primer template (Fig. 1h). We suspect that the DNA structure or RecA, which are unique to the D-loop extension reaction, contribute to the reduced fidelity of Pol IV. Previous studies showed that RecA interacts with Pol IV and collaborates with UmuD to modulate the fidelity of the polymerase 29. Thus, RecA binding to Pol IV could conceivably reduce its ability to discriminate against incorrect nucleotides during RDR. Alternatively, the dynamic structure of the D loop may affect the fidelity of Pol IV. For example, RecA-mediated D loops are unstable because of the ability of the recombinase to promote dissociation of the invading strand 41. Thus, the inherent instability of the D loop may compromise the fidelity of all polymerases. This would indicate that RDR is inherently error prone. However, considering that error-prone recombination has not been observed in proliferating E. coli cells, other factors may be needed to promote high-fidelity RDR. For example, mismatch repair proteins that ensure accurate recombination and replication may prevent error-prone RDR in the absence of stress. Consistent with this, suppression of mismatch repair during stationary phase is thought to contribute to error-prone recombination 18. Further studies are needed to determine what reduces the accuracy of Pol IV at D loops, whether this phenomenon is universal for all polymerases and whether other factors promote high-fidelity RDR.

Genetic studies have shown that SOS-induced levels of Pol IV are necessary for its involvement in error-prone recombination 31. This suggests that upregulation of Pol IV promotes its RDR activity. In line with the previous genetics data, we have demonstrated that SOS-induced concentrations of Pol IV facilitate its recruitment to D loops (Fig. 2). For example, at concentrations corresponding to nonstressed conditions, PriA, which facilitates replisome assembly at D loops, blocked Pol IV RDR activity for the most part. Yet at concentrations corresponding to stressed-induced conditions, Pol IV outcompeted PriA at D loops. We further found that Pol IV outcompeted Pol I (Supplementary Fig. 6), Pol II (Fig. 5) and the Pol III replisome (Supplementary Fig. 3) at polymerase concentrations relevant to stressed-induced cells. Although previous genetics data have implied competition between Pol IV and these other polymerases 20, our data suggest that Pol IV is preferentially recruited to D loops under stress-induced conditions, probably facilitating error-prone recombination.

Previous in vivo studies have shown that the exonuclease domain of Pol II reduces (~83%) stress-induced mutations by an unknown mechanism 32. In an effort to elucidate this regulatory function of Pol II, we examined its activity and ability to compete with Pol IV during RDR. We found that the exonuclease activity of Pol II is markedly stimulated during D-loop extension, even at saturating dNTP concentrations (~50 µM) (Fig. 3). This D loop–dependent stimulation causes Pol II to switch to an active exonuclease mode during RDR, which is promoted by topological stress (positive supercoils) and reduced dNTP pool concentrations during stationary phase (Fig. 3). However, Pol II reduces (~83%) stress-induced mutations by an unknown mechanism, another factor may be needed to promote high-fidelity RDR. For example, mismatch repair proteins that ensure accurate recombination and replication may prevent error-prone RDR in the absence of stress. Consistent with this, suppression of mismatch repair during stationary phase is thought to contribute to error-prone recombination 18. Further studies are needed to determine what reduces the accuracy of Pol IV at D loops, whether this phenomenon is universal for all polymerases and whether other factors promote high-fidelity RDR.

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Although gyrase reduces positive supercoils in DNA, we found that the topoisomerase only delays the exonuclease mode of Pol II in our assay (Supplementary Fig. 5). This suggests that the exonuclease activity of Pol II may be similarly stimulated at D loops in vivo.

We also found that the exonuclease activity of Pol II enables it to compete with Pol IV at D loops (Fig. 4). Although Pol II is capable of promoting a small fraction of stress-induced mutations, it performs high-fidelity DNA synthesis compared to Pol IV. Thus, the ability of exonuclease-proficient Pol II to compete with Pol IV and presumably correct Pol IV errors provides an explanation of why the exonuclease domain of Pol II suppresses stress-induced mutations in vivo.

Cellular studies have demonstrated that both the SOS andRpS stress responses are necessary for Pol IV involvement in error-prone recombination. Upregulation of Pol IV (to ~2,500 molecules per cell) by the SOS response is thought to be the sole role of this stress response in error-prone recombination.11 Consistent with this, we have demonstrated that levels of Pol IV corresponding to those in SOS-induced cells facilitate its recruitment to D loops (Fig. 2). However, the RpoS stress response, which is activated during growth-limiting conditions, contributes to Pol IV RDR activity has remained unclear. We showed that an additional 100% increase in Pol IV levels, as is observed during the RpoS stress response, enables Pol IV to outcompete Pol II, resulting in a net reduction in D-loop resection by Pol II (Fig. 5)17. Thus, our findings indicate that upregulation of Pol IV by the RpoS response contributes to its role in error-prone RDR. The RpoS stress response may, however, elicit other factors or processes that facilitate Pol IV involvement in RDR. For example, the possibility exists that Pol IV undergoes post-translational modifications that promote its RDR activity.

This report provides insight into the mechanism and regulation of error-prone recombination during stress (Fig. 6). Our data suggest that Pol IV has a dominant role in RDR during stress that is caused by its upregulation by the SOS and RpoS responses. Preferential D-loop extension by Pol IV during stress probably facilitates error-prone RDR, which allows E. coli to rapidly evolve and overcome adverse environmental conditions such as exposure to antibiotics.16,19 We propose that Pol IV RDR errors are suppressed by Pol II through its active exonuclease domain, enabling it to compete with Pol IV and delete Pol IV errors by partially resecting the extended D loop. However, because Pol IV is highly abundant in stressed cells, it probably regains access to the D loop by displacing Pol II from DNA. This model supports a dynamic interplay among translesion polymerases at D loops during stress and explains how the exonuclease domain of Pol II reduces stress-induced mutations in vivo.35

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.T.P. conceived the idea for the study, wrote the manuscript and performed and interpreted all experiments, with the exception of those in Figure 3i, which were performed by I.K., M.E.O. and M.F.G. provided editorial input.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Cox, M.M. Recombinational DNA repair of damaged replication forks in Escherichia coli: questions. Annu. Rev. Genet. 35, 53–82 (2001).
2. Cox, M.M. et al. The importance of repairing stalled replication forks. Nature 404, 37–41 (2000).
3. Mazon, G., Mimitou, E.P. & Symington, L.S. SnapShot: homologous recombination in DNA double-strand break repair. Cell 142, 646, 646.e1 (2010).
4. Li, X. & Heyer, W.D. Homologous recombination in DNA repair and DNA damage tolerance. Cell Res. 18, 99–113 (2008).
5. Heller, R.C. & Marians, K.J. Replisome assembly and the direct restart of stalled replication forks. Nat. Rev. Mol. Cell Biol. 7, 932–943 (2006).
6. Zahradka, K. et al. Reassembly of shattered chromosomes in Deinococcus radiodurans. Nature 443, 569–573 (2006).
7. Motamedi, M.R., Szigety, S.K. & Rosenberg, S.M. Double-strand-break repair recombination in Escherichia coli: physical evidence for a DNA replication mechanism in vivo. Genes Dev. 13, 2889–2903 (1999).
8. Lyedard, J.R. et al. Break-induced replication requires all essential DNA replication factors except those specific for pre-RC assembly. Genes Dev. 24, 1133–1144 (2010).
9. Kawamoto, T. et al. Dual roles for DNA polymerase η in homologous DNA recombination and translesion DNA synthesis. Mol. Cell 20, 793–799 (2005).
10. Kane, D.P., Shusterman, M., Rong, Y. & McVey, M. Competition between replicative and translesion polymerases during homologous recombination repair in Dragophila. PLoS Genet. 8, e1002659 (2012).
11. Li, X., Stith, C.M., Burgers, P.M. & Heyer, W.D. PCNA is required for initiation of replication forks. Nat. Struct. Mol. Biol. 19, 17–24 (2012).
12. Moldovan, G.L. et al. DNA polymerase POLN participates in cross-link repair and homologous recombination. Mol. Cell Biol. 30, 1088–1096 (2010).
13. Michalowicz, M.J. et al. Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination. Mol. Cell 20, 783–792 (2005).
14. Kohzaki, M. et al. DNA polymerases η and δ are required for efficient immunoglobulin V gene diversification in chicken. J. Cell Biol. 189, 1117–1127 (2010).
15. Rosenberg, S.M. Evolving responsively: adaptive mutation. Nat. Rev. Genet. 2, 504–515 (2001).
16. Ponder, R.G., Fonville, N.C. & Rosenberg, S.M. A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. Mol. Cell 19, 791–804 (2005).
18. McKenzie, G.J., Lee, P.L., Lombardo, M.J., Hastings, P.J. & Rosenberg, S.M. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Mol. Cell 7, 571–579 (2001).

19. Foster, P.L. Stress-induced mutagenesis in bacteria. Crit. Rev. Biochem. Mol. Biol. 42, 373–397 (2007).

20. Hastings, P.J. et al. Competition of Escherichia coli DNA polymerases I, II and III with DNA Pol IV in stressed cells. PLoS ONE 5, e10862 (2010).

21. Shee, C., Gibson, J.L. & Rosenberg, S.M. Two mechanisms produce mutation hotspots at DNA breaks in Escherichia coli. Cell Rep. 2, 714–721 (2012).

22. Harris, R.S., Bull, H.J. & Rosenberg, S.M. A direct role for DNA polymerase III in adaptive reversion of a frameshift mutation in Escherichia coli. Mutat. Res. 375, 19–24 (1997).

23. Schlacher, K., Cox, M.M., Woodgate, R. & Goodman, M.F. RecA acts in trans to allow replication of damaged DNA by DNA polymerase V. Nature 442, 883–887 (2006).

24. Petrosino, J.F., Galhardo, R.S., Morales, L.D. & Rosenberg, S.M. Stress-induced β-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the Escherichia coli chromosome. J. Bacteriol. 191, 5981–5989 (2009).

25. Kim, S.R., Matsui, K., Yamada, M., Gruz, P. & Nohmi, T. Roles of chromosomal and episomal dinB genes encoding DNA pol IV in targeted and untargeted mutations in the Escherichia coli chromosome. J. Bacteriol. 191, 5981–5989 (2009).

26. Tippin, B., Pham, P. & Goodman, M.F. Error-prone replication for better or worse. Trends Microbiol. 12, 288–295 (2004).

27. Akerlund, T., Nordstrom, K. & Bernarder, R. Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of Escherichia coli. J. Bacteriol. 177, 6791–6797 (1995).

28. Withers, H.L. & Bernarder, R. Characterization of dnaC2 and dnaC28 mutants by flow cytometry. J. Bacteriol. 180, 1624–1631 (1998).

29. Godoy, V.G. et al. UmuD and RecA directly modulate the mutagenic potential of the Y family DNA polymerase DinB. Mol. Cell 28, 1058–1070 (2007).

30. Kobayashi, S., Valentine, M.R., Pham, P., O’Donnell, M. & Goodman, M.F. Fidelity of Escherichia coli DNA polymerase IV. Preferential generation of small deletion mutations by dNTP-stabilized misalignment. J. Biol. Chem. 277, 34198–34207 (2002).

31. Galhardo, R.S. et al. DinB upregulation is the sole role of the SOS response in stress-induced mutagenesis in Escherichia coli. Genetics 182, 55–68 (2009).

32. Xu, L. & Marians, K.J. PriA mediates DNA replication pathway choice at recombination intermediates. Mol. Cell 11, 817–826 (2003).

33. Shlima, J. & Korlhuber, A. A prepriming DNA replication enzyme of Escherichia coli. II. Purification of protein n2, a sequence-specific, DNA-dependent ATPase. J. Biol. Chem. 255, 6789–6793 (1980).

34. Frisch, R.L. et al. Separate DNA Pol II– and Pol IV–dependent pathways of stress-induced mutation during double-strand-break repair in Escherichia coli are controlled by RpoS. J. Bacteriol. 192, 4634–4700 (2010).

35. Foster, P.L., Gudmundsson, G., Trimmarchi, J.M., Cai, H. & Goodman, M.F. Proofreading-defective DNA polymerase II increases adaptive mutation in Escherichia coli. Proc. Natl. Acad. Sci. USA 92, 7951–7955 (1995).

36. Bonner, C.A. et al. Purification and characterization of an inducible Escherichia coli DNA polymerase capable of insertion and bypass at abasic lesions in DNA. J. Biol. Chem. 263, 18946–18952 (1988).

37. Cai, H., Yu, H., McEntee, K., Kunkel, T.A. & Goodman, M.F. Purification and properties of wild-type and exonuclease-deficient DNA polymerase II from Escherichia coli. J. Biol. Chem. 270, 15327–15335 (1995).

38. Goodman, M.F. et al. DNA Replication and Mutagenesis (American Society for Microbiology, Washington, D.C., 1988).

39. Buckstein, M.H., He, J. & Rubin, H. Characterization of nucleotide pools as a function of physiological state in Escherichia coli. J. Bacteriol. 190, 718–726 (2008).

40. Ibarra, B. et al. Proofreading dynamics of a processive DNA polymerase. EMBO J. 28, 2794–2802 (2009).

41. Downey, C.D. & McHenry, C.S. Chaperoning of a replicative polymerase onto a newly assembled DNA-bound sliding clamp by the clamp loader. Mol. Cell 37, 481–491 (2010).

42. Baker, T. & Korlhuber, A. DNA Replication, 2nd edn (University Science Books, 1992).

43. Lovett, S.T. Replication arrest-stimulated recombination: dependence on the RecA paralog, RadA/Sms and translesion polymerase, DinB. DNA Repair (Amst.) 5, 1421–1427 (2006).

44. Register, J.C. III & Griffith, J. Direct visualization of RecA protein binding to and unwinding duplex DNA following the D-loop cycle. J. Biol. Chem. 263, 11029–11032 (1988).
ONLINE METHODS

Recombination-directed replication. Sixteen-micromolar (in nucleotides) 32P-labeled ssDNA was incubated with 5.2 μM RecA, 0.5 mM ATP, 40 mM phosphocreatine, 1 μg creatine phosphokinase and 200 μM dNTPs in a total volume of 5 μl of buffer A (25 mM Tris-HCl, pH 7.5, 0.1 mg/ml BSA, 1 mM DTT and 15 mM MgCl2) for 5 min. The reaction was then mixed with 5 μl of buffer A containing 740 μM (in nucleotides) supercoiled pRP27, 0.5 mM ATP, 40 mM phosphocreatine and 1 μg creatine phosphokinase for a further 1.5 min. Then the reaction was mixed with 10 μl of buffer A containing 0.5 mM ATP, 0.2 μM γ complex, 2.6 μM SSB and 1 μM β for 1 min. Polymerase was then added for the times indicated. In Figures 2 and 3c and Supplementary Figures 2, 4 and 5, polynucleotides were added along with complex, SSB, β and other factors (PriA, RuvAB and gyrase). γ complex was replaced with τ complex in Figures 2a and 3h and Supplementary Figure 2. Reactions were terminated by the addition of 20 mM EDTA, 2 mg/ml proteinase K and 0.6% SDS and incubated for a further 15–30 min. Reaction products were resolved in nondenaturing agarose gels, except for those in Figure 1h–j, which were purified twice through microspin S–400 homologous recombination columns (GE Healthcare) and then resolved in denaturing urea polyacrylamide gels. Reaction products were analyzed using a phosphorimager (Typhoon 9400, GE Healthcare, Life Sciences). The reactions in Figure 1a included a 3.3 μM final concentration of RecA and the indicated concentrations of trans ssDNA (RP158). Reactions were performed at 37 °C. The polymerase concentrations used were as follows, except where indicated in the figures: Pol III, 50 nM; Pol I, 1 μM; Pol II, 875 nM; and Pol IV SOS concentrations, 6.25 μM, which were purified twice through microspin Maxiprep kit and then centrifuged at 90,000 g. RecA, Pol III, Pol IV, Pol V, Gyrase were purchased from New England Biolabs.

Pol II replication of a primer template. Thirty-nanomolar Pol II (lanes 8–12) or Pol III (lanes 2–6) was added, and aliquots of the reactions were terminated at the indicated time intervals by the addition of 25 μM of stop buffer (40 mM HEPES, pH 7.5, 40 mM EDTA and 6% SDS). DNA was released from beads by incubating at 95 °C for 3 min and then resolved in 0.8% denaturing alkali agarose gels.

Pol III replication of a primer template. Reactions contained 37.5 fmol primed M13mp18 ssDNA, 60 μM dCTP and dGTP, 0.5 mM ATP, 0.5 μM SSB, 350 fmol β, and 100 fmol Pol III* or Pol III* containing an ε mutant (D12A and E14A) that eliminates the 3→5′ exonuclease activity in 22 μl replication buffer (20 mM Tris-HCl, pH 7.5, 4% glycerol, 0.1 mM EDTA, 40 μg/ml BSA, 5 mM DTT and 15 mM MgCl2) for 5 min (Supplementary Fig. 3). The reaction was then mixed with 5 μl of buffer B containing 925 μM (in nucleotides) supercoiled pRP27, 0.5 mM ATP, 40 mM phosphocreatine, 1 μg creatine phosphokinase, 1 μCi 32P–dATP and 7 μM SSB for a further 1.5 min. Then the reaction was mixed with 15 μl of buffer B containing (0.5 mM ATP) 85 nM Pol III*, 0.85 μM β, 17 or 34 nM of PriA, PriB and PriC (PriA/B/C), 612 nM DnaB, 714 nM DnaC, 680 nM DnaG, 306 nM DnaT, 25.5 μM gyrase, 340 μM NTPs and the indicated final concentrations of Pol IV for 40 min. Reactions were terminated by the addition of 20 mM EDTA, 2 mg/ml proteinase K and 0.6% SDS and incubated for a further 15–30 min. Reaction products were resolved in nondenaturing agarose gels (and in an alkaline agarose gel, where indicated) and analyzed by phosphorimager. Reactions were performed at 37 °C.

Exonuclease activity assay. Ten-nanomolar 32P-labeled primer template (25/10 or 25G/10 (containing mismatch)) was mixed with 8 mM MgCl2, 200 nM β, 60 nM γ complex, 1 mM ATP and 1 mM SSB in buffer C (20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 5 mM DTT and 10% glycerol) for 2 min (Fig. 3k). Thirty-nanomolar Pol II (lanes 8–12) or Pol III (lanes 6–9) was added, and aliquots of the reactions were terminated at the indicated time intervals by the addition of 25 mM EDTA and 45% formamide. Reactions were performed at 37 °C. Products were resolved in a urea polyacrylamide gel and analyzed by phosphorimager.

Proteins. Pol III (WT and exonuclease deficient), Pol IV, Pol V, β and the reconstituted γ complex were purified as described33,35,36. The τ complex was reconstituted from pure proteins and purified in an identical fashion as the γ complex except that γ was replaced with τ. Histidine-tagged versions of PriA/B/C and DnaT were purified by standard Ni2+ affinity chromatography methods. DnaG, DnaB and DnaC were purified as described46,47. WT and exonuclease-deficient Pol II were purified as described37,48. RuvA and RuvB were purified as described49. RecA, Pol I (Klenow fragment and Klenow fragment 3′→5′ exonuclease deficient) and Gyrase were purchased from New England Biolabs.

DNA. Supercoiled plasmid DNA (pRP27) was purified using a Qiagen Maxiprep kit and then centrifuged at 90,000g for 48 h at 20 °C through a cesium chloride density gradient. 100mer rolling circle DNA was purified.
50. Primed M13mp18 ssDNA was prepared as described\textsuperscript{45}. The linear dsDNA template was prepared as described\textsuperscript{51}. The oligonucleotides used were as follows (5′→3′): RP192, TTCGTCT TCAAGAATCTCTGTTTTGACAGCTTATCATCGATCTGCAATATACGAATTCAGGAGG GAGATGAGAGAATTGGG; RP158, GGTACGCGATCAATCAGCTGAGACCGCAA ATACGGATAAGGGC TGAGCAGCTCTGCGACGCTGCGAGCTGCGAATCTCTG; RP219A, AAGAAAACCATTATTTATCATGACATATTACCTTAAATAGGCGTAT CACGAGGCCCTTTCGTCCTTAAGATA; RP25, CACAGATTCTCGCGA GGCTGCAGATCGC; RP10, AGCTGAGACCGCAAATACGGATAAGGGC TGAGCAGCTCTGCGACGCTGCGAGCTGCGAATCTCTG; RP25G, CACAGATTCTCGCGACGCTGCGAGCTGCGAATCTCTG; RP312, TCACGAGGCCCTT TCCTCTCAAAAT; RP313, TGTCAACATGAGAAATTTCTGAGAAC GAAAGGGCCCTCGTA; RP235A, TTTGACAGCTTATCATCGATCTGC AGTAATACGACTCACAATAGGAGGAGGAGGATGAGAATAT; RP235AM1, TTTGACAGCTTATCATCGATCTGCAATACGACTCA CTATAGGAGGAGGATGAGAATATG; and RP235AM2, TTTGACAGCTTATCATCGATCTGCAATACGACTCACTATAGGGA GGAGGAGGATGAGAATATGGG.

\textsuperscript{45} Indiani, C., McInerney, P., Georgescu, R., Goodman, M.F. & O’Donnell, M. A sliding-clamp toolbelt binds high- and low-fidelity DNA polymerases simultaneously. Mol. Cell \textbf{19}, 805–815 (2005).

\textsuperscript{46} Georgescu, R.E. \textit{et al.} Mechanism of polymerase collision release from sliding clamps on the lagging strand. \textit{EMBO J.} \textbf{28}, 2981–2991 (2009).

\textsuperscript{47} Davey, M.J., Fang, L., McInerney, P., Georgescu, R.E. & O’Donnell, M. The DnaC helicase loader is a dual ATP/ADP switch protein. \textit{EMBO J.} \textbf{21}, 3148–3159 (2002).

\textsuperscript{48} Indiani, C., Langston, L.D., Yurieva, O., Goodman, M.F. & O’Donnell, M. Translesion DNA polymerases remodel the replisome and alter the speed of the replicative helicase. \textit{Proc. Natl. Acad. Sci. USA} \textbf{106}, 6031–6038 (2009).

\textsuperscript{49} Kaplan, D.L. & O’Donnell, M. RuvA is a sliding collar that protects Holliday junctions from unwinding while promoting branch migration. \textit{J. Mol. Biol.} \textbf{355}, 473–490 (2006).

\textsuperscript{50} McInerney, P. & O’Donnell, M. Functional uncoupling of twin polymerases: mechanism of polymerase dissociation from a lagging-strand block. \textit{J. Biol. Chem.} \textbf{279}, 21543–21551 (2004).

\textsuperscript{51} Pomerantz, R.T. & O’Donnell, M. The replisome uses mRNA as a primer after colliding with RNA polymerase. \textit{Nature} \textbf{456}, 762–766 (2008).