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Preferential D-loop Extension by a Translesion DNA Polymerase Underlies Error-Prone Recombination

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Summary

Although homologous recombination (HR) is considered an accurate form of DNA repair, genetics suggest that *Escherichia coli* (*E. coli*) translesion DNA polymerase (pol) IV (DinB) promotes error-prone recombination during stress which allows cells to overcome adverse conditions. How pol IV functions and is regulated during recombination under stress, however, is unknown. We show that pol IV is highly proficient in error-prone recombination, and is preferentially recruited to D-loops at stress-induced concentrations in vitro. Unexpectedly, we find that high-fidelity pol II switches to exonuclease mode at D-loops which is stimulated by topological stress and reduced deoxy-ribonucleotide pools observed during stationary-phase. The exonuclease activity of pol II enables it to compete with pol IV which likely 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 (HR) repairs double-strand breaks (DSBs) by directing replication to copy sequence information from a homologous donor (Fig. 1a)[1–4]. For example, following formation of a DSB, nucleases resect the DNA resulting in a 3' single-strand DNA (ssDNA) tail. RecA type recombinases form a filament along the tail which facilitates strand invasion within a homologous donor DNA resulting in a displacement loop (D-loop). DNA polymerase (pol) then extends the 3' end of the invading strand by 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.

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Importantly, the accuracy of HR, which is widely considered to be high, is dependent on the fidelity of RDR. Although high-fidelity replicative pols predominantly perform RDR[5–8], mounting evidence indicates that low-fidelity translesion pols also carry out this function which suggests that HR is error-prone. For example, previous studies in eukaryotes including humans, yeast, chickens, frogs and flies indicate that low-fidelity translesion pols η, ν, θ, and ζ contribute to RDR which probably promotes mutagenesis[9–15]. Moreover, recent evidence from Drosophila suggests that translesion pols even compete with replicative pols during HR[10]. Although translesion pols are widely known to promote replication past lesions in DNA, it is becoming clear that these low-fidelity enzymes function during HR 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 Y-family translesion pol IV (DinB) promotes error-prone HR in the form of mutations specifically during stress[16–20]. Such error-prone recombination allows *E. coli* to rapidly evolve and overcome stressful conditions including nutritional starvation and exposure to antibiotics[16,19]. 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 HR is mostly based 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 (i.e. RecA, RecBCD, RuvABC), and the RpoS stress response which further upregulates pol IV (~100%) and downregulates mismatch repair[16,17,19,21,22]. Current models based on these findings propose that pol IV promotes mutations near D-loops during HR under stress[17,20]. 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 pols during HR under stress remains unclear. Here we sought to provide mechanistic insight into the activity and regulation of pol IV during HR 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 that pol IV promotes RDR (Fig. 1c, lanes 1–4), which requires the polymerase and RecA (Fig. 1d). In contrast, the related Y-family pol V (UmuD'$_2$C) failed to perform RDR under identical conditions (Fig. 1c, lanes 5–8). The inability of pol V to promote RDR was surprising since its activity requires RecA filaments *in trans* which are present in excess as indicated by the free ssDNA (Fig. 1c, lower band)[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. Still, pol V failed to promote RDR (Fig. 1e). As a positive control, we demonstrated that the same amount of pol V extends a simple primer-template which requires ssDNA and RecA *in trans* as shown previously (Fig. 1f)[23]. Considering pol V plays a small role in promoting stress-induced mutations during recombination, additional factors may be needed to stimulate its activity at D-loops[24].

We next examined whether β, which confers processivity onto pols, is required for pol IV RDR activity. Here a relatively high polymerase to D-loop ratio was used which we based on approximate conditions in SOS-induced cells (Table 1). For example, pol IV is highly upregulated (~10^1) by the SOS response to ~2,500 molecules per cell, making it the most abundant DNA polymerase in stressed cells (Table 1)[25,26]. Since 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 since previous estimates suggest that each chromosome incurs a break and *E. coli* contains four chromosomes in rich medium[2,27,28]. Since 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 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 and is in contrast to previous biochemical studies of yeast proteins which have demonstrated that PCNA, the eukaryotic equivalent of β, is required for RDR by replicative pol δ and translesion pol η[11]. Our results suggest that pol IV may not require β for its involvement in RDR due to its abundance in stressed cells.

Next, we 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 pols 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 which 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 exhibited a surprisingly high efficiency of nucleotide misincorporation on a D-loop under similar conditions and sequence context as in the previous experiment with the primer-template (Fig. 1h, compare right and left panels). Pol IV also appears to be more prone to mismatch extension on the D-loop which is indicated by the upper bands in lanes two and four (Fig. 1h, right). These data suggest that RDR may be inherently error-prone. Alternatively, since 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[29].

Considering 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. Comparison of pol IV activity on D-loops with different sequences suggests 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 1i). For example, in the right panel of figure 1h pol IV preferentially misincorporated dCMP which is complementary to the +2 template base guanosine. In contrast, dGMP is preferentially misincorporated in figure 1i where cytosine is the +2 template base. Previous biochemical studies have clearly shown that pol IV is able to ‘skip

over' the correct template base which facilitates a −1 frameshift mutation[30]. The ability of pol IV to promote −1 frameshifts has been widely used to detect its activity in error-prone recombination[16,18,19]. Consistent with these previous studies, we showed that pol IV is capable of promoting a −1 frameshift on a D-loop (Fig. 1j). Importantly, pol IV induced mutations are likely to go unrepaired during stationary-phase since mismatch repair is deficient[16,19]. The data presented in figure 1 demonstrate that pol IV is highly proficient in error-prone RDR which provides mechanistic insight into its role in stress-induced mutagenesis[18].

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[5]. Since 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[31]. For example, polymerase access to D-loops in proliferating cells is prevented by primosomal protein PriA which binds tightly to D-loops where it recruits other primosomal proteins to assemble the replisome during RDR (Fig. 2a, left)[32]. Consistent with previous studies, PriA inhibited D-loop extension by replicative pol III (Fig. 2a, right)[32]. Similarly, concentrations of PriA corresponding to known amounts in the cell (~70 molecules per cell[33]) suppressed RDR by pol IV at levels corresponding to non-stressed cells (~250 molecules per cell[25,26]) (Fig. 2b, left panel). In contrast, PriA only slightly inhibited D-loop extension by pol IV when the polymerase was added at levels comparable to stress-induced concentrations (~2,500 molecules per cell; Fig. 2b, right panel). This indicates that pol IV outcompetes PriA at D-loops during stress (Fig. 2b, right schematic). Similar preferential RDR activity by pol IV was observed in the presence of RuvAB which also binds tightly to D-loops and promotes stress-induced mutations (Supplementary Fig. 2)[16]. We further found that pol IV levels comparable to SOS-induced cells promote RDR in the presence of all the primosomal and replisome components and supersede D-loop dependent replisome activity (Supplementary Fig. 3). Together, these results indicate that pol IV gains access to D-loops and overrides the primosome dependent RDR machinery during stress.

D-loop dependent stimulation of pol II exonuclease activity

Genetic data suggest that translesion pol II also plays a role in RDR during growth-limiting conditions[20,34,35]. Similar to pol IV, pol II is upregulated during stress (~350 molecules per cell, Table 1[36]) and is considered a translesion pol due to its ability to promote replication past certain lesions[26]. However, unlike pol IV which is a Y-family DNA polymerase that exhibits low-fidelity DNA synthesis, pol II is among the B-family of pols which exhibit high-fidelity DNA synthesis and exonuclease activities. Pol II therefore possesses similar characteristics to eukaryotic replicative pols δ and ε which are also B-family members. Interestingly, genetic studies indicate that the exonuclease domain of pol II suppresses stress-induced mutations[35]. Considering pol IV promotes most stress-induced mutations, this suggests that pol II may regulate pol IV activity during RDR by competing with and proofreading pol IV errors.

We investigated whether pol II performs RDR in figure 3. Remarkably, we found that although pol II initially extends the D-loop, the reaction is subsequently reversed, presumably due to the enzyme's 3'–5' 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 the reaction was performed with a saturating deoxy-ribonucleotide (dNTP) concentration (50 μM) that exceeds the enzyme's Km 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 likely relevant to pol II activity in stressed cells and thus warranted further investigation.

We hypothesized that the exonuclease domain of pol II is stimulated due to 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 and exonuclease deficient pol II act similar on a linear double-strand DNA template and a circular primer-template which indicates 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), which appears to be due to a slight delay in pol II exonuclease activity (Supplementary Fig. 5). These results support a model whereby superhelical tension generated during D-loop extension promotes the reverse translocation and exonuclease function of pol II which facilitates a switch to a highly active exonuclease mode (Fig. 3d). This model is supported by biophysical studies that have demonstrated that mechanical tension on the DNA template stimulates proofreading by bacteriophage phi29 pol[40]. We further found that reducing the dNTP pool to 10 μM facilitates the reverse reaction (compare Fig. 3e with Fig. 3a, left), whereas increasing the dNTP pool to 100 μM—which reflects the conditions in proliferating cells—either during (Fig. 3f) or prior (Fig. 3g) to the reaction prevents pol II from switching to exonuclease mode during the same timecourse[39]. These findings suggest that the enzyme's Km for dNTPs is increased under conditions of opposing force (i.e. superhelical tension).

We next examined whether this exonuclease activity is specific to pol II by comparing RDR by wildtype and exonuclease deficient pol III. Here, similar conditions were used as with pol II (i.e. 50 μM dNTPs), however, the γ-subunit of the clamp-loader was substituted with τwhich specifically binds to pol III and facilitates its recruitment to DNA; τrepresents 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 wild-type pol III (Fig. 3h), but acts the same as wild-type on a primer-template (Fig. 3i) and within the replisome (Fig. 3j). Hence, the exonuclease domain of pol III was also surprisingly activated at D-loops. However, only pol II exhibited the unique ability to reverse the D-loop extension reaction which we attribute to its highly active exonuclease domain compared to 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 observed in SOS-induced cells (see Table 1). Since 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 during RDR as indicated by a net increase in D-loop extension by pol IV versus D-loop resection by pol II (Fig. 4a, left; compare lanes 7–9 to lanes 1–3 and 4–6). Further reduction of the dNTP pool (10 μM) enhanced pol IV activity compared to pol II (Fig. 4a, right). Since the exonuclease function of pol II reduces (~83%) stress-induced mutations in vivo[35], we wondered whether the observed exonuclease activity affects the ability of pol II to compete with pol IV. To test this idea, we examined competition between exonuclease deficient pol II and pol IV during RDR. The different D-loop extension products indicated that exonuclease deficient pol II fails to effectively compete with pol IV (Fig. 4b, left and right panels; compare lanes 7–9 to lanes 1–3 and 4–6). Considering similar products were generated at 50 μM dNTPs (left), we further resolved the polymerases' products which unequivocally showed that exonuclease deficient pol II is unable to effectively compete with pol IV (Fig. 4c). These results were in contrast to those observed for wild-type pol II which competes with pol IV (Fig. 4a). Thus, the data in figure 4 demonstrate the unexpected finding that pol II requires a functional exonuclease domain to compete with pol IV and thus regulate error-prone RDR.

Importantly, the concentration of pol IV is further upregulated approximately 100 percent (to ~5,000 molecules per cell) by the RpoS general stress response in stationary-phase cells[17,20]. Whether this increase in pol IV expression contributes to its activity at D-loops, however, is unknown. We found that increasing the relative levels of pol IV by 100 percent enabled pol IV to further compete with pol II as indicated by a marked reduction in net exonuclease activity at D-loops (compare Fig. 5 with Fig. 4a). Considering pol IV outcompeted pol II, the pol III replisome (Supplementary Fig. 3) and pol I (Supplementary Fig. 6) which is the second most abundant pol in stressed cells (~400 molecules per cell[42]), our data suggest that pol IV plays a major role in RDR during stress which likely facilitates adaptive evolution.

Discussion

Models based on genetics have long proposed a role for pol IV in error-prone RDR in growth-limited cells as the mechanism of stress-induced mutagenesis, also known as error-prone recombination[16,17,19]. Genetics also implicate pol IV in RDR during replication restart[43]. In vitro evidence supporting pol IV RDR activity, however, has been lacking. This report verifies the ability of pol IV to promote RDR in a reconstituted assay which, in conjunction with previous genetics, 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 which is consistent with its ability to generate mutations during HR 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[17,20]) provide an explanation of why this enzyme is responsible for most (85%) stress-induced mutations[18].

Intriguingly, 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 due to the ability of the recombinase to promote dissociation of the invading strand[44]. Thus, the inherent instability of the D-loop may compromise the fidelity of all pols. This would indicate that RDR is inherently error-prone. However, considering 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 which 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 errorprone 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 pols, 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 errorprone recombination[31]. This has suggested that upregulation of pol IV promotes its RDR activity. Inline with the previous genetics, we have demonstrated that SOS-induced concentrations of pol IV facilitate its recruitment to D-loops (Fig. 2). For example, at concentrations corresponding to non-stressed conditions, PriA which facilitates replisome assembly at D-loops mostly blocked pol IV RDR activity. 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 have implied competition between pol IV and these other pols[20], our data suggest that pol IV is preferentially recruited to D-loops under stress-induced conditions which likely facilitates 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[35]. In an effort to elucidate this regulatory function of pol II, we examined its activity and ability to compete with pol IV during RDR. Remarkably, we found that the exonuclease activity of pol II is dramatically 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 (i.e. positive supercoils) and reduced dNTP pools observed during stationary-phase (Fig. 3). 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 surprisingly 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[20,34], 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.

Lastly, cellular studies have demonstrated that both the SOS and RpoS stress responses are necessary for pol IV involvement in error-prone recombination[17]. 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[31]. Consistent with this, we have demonstrated that levels of pol IV corresponding to SOS-induced cells facilitate its recruitment to D-loops (Fig. 2). How the RpoS stress response which is activated during growth-limiting conditions contributes to pol IV RDR activity, however, has remained unclear. We showed that an additional 100 percent increase in pol IV levels which is observed during the RpoS stress response enables pol IV to outcompete pol II which results 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 may undergo post-translational modifications that promote its RDR activity.

In summary, this report provides unexpected insight into the mechanism and regulation of error-prone recombination during stress and supports the model illustrated in figure 6. Our data suggest that pol IV plays a dominant role in RDR during stress which is due to its upregulation by the SOS and RpoS responses. Preferential D-loop extension by pol IV during stress likely 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 which enables it to compete with pol IV and delete pol IV errors by partially resecting the extended D-loop. However, since pol IV is highly abundant in stressed cells, it likely regains access to the D-loop by displacing pol II from DNA. This model supports a dynamic interplay among translesion pols at D-loops during stress and explains how the exonuclease domain of pol II reduces stress-induced mutations in vivo[35].

Online Methods

Recombination-directed replication

16 μM (in nucleotides) ^{32}P-5' 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 TrisHCl pH 7.5, 0.1 mg/ml BSA, 1 mM DTT, 15 mM MgCl) 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. Next, 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_2 for 1 min. Pol was then added for the times indicated. In figures 2, 3c, and supplementary figures 2, 4 and 5, pols were added along with γ-complex, SSB, β and other factors (PriA, RuvAB, Gyrase). γ-complex was replaced with τ-complex in figures 2a, 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 non-denaturing agarose gels except for those in figures 1h–j which were purified twice through microspin S-400 HR columns (GE Healthcare) then resolved in denaturing urea polyacrylamide gels. Reaction products were analyzed by phosphorimager. Reactions in figure 1e included 3.3 μM final concentration of RecA and the indicated concentrations of *trans* ssDNA (RP158). Reactions were performed at 37 °C. Concentrations of pols used were as follows except where indicated in figures: pol III (50 nM); pol I (1 μM); pol II (875 nM); pol IV (SOS concentrations, 6.25 μM; non-SOS concentrations, 625 nM). RP192 ssDNA was used in all experiments except for figure 1h (RP219A), and figures 1i,j (RP235A). ssDNA was 5'-end labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase (New England Biolabs).

Primer-template extension assays. (Fig. 1f)

Primer extension by pol V with RecA and *trans* ssDNA was performed as described[23], however, a different primer-template (RP158/RP25) was used and the concentration of pol V was 500 nM. Products were resolved in a denaturing urea polyacrylamide gel and visualized by phosphorimager. The primer-template was assembled by mixing equimolar concentrations of RP25 and RP158 followed by heating to 90–100 °C then slowly cooling to room temp. (Fig. 1h) Primer extension was performed by incubating 500 nM β, 100 nM γ-coplex, and 1.3 μM SSB with 40 nM primer-template (RP312/313) and 50 μM of the indicated dNTP in buffer A at 37° C for 1 min followed by the addition of 500 nM pol IV for an additional 5 min. Assembly of the primer-template and resolution of the reaction products were performed as above. Primers were 5'-end labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase (New England Biolabs).

Pol II replication of double-strand DNA (Figure 3b, left)

10 nM linear double-strand DNA containing a replication fork at the 5' end was mixed with 100 nM γ-complex, 500 nM β, 50 μM dNTPs, 2 mM ATP, 3 μCi ^{32}P-α-dATP and 1.3 μM SSB for 1 min at 37° C in buffer A. 875 nM wild-type or exonuclease deficient pol II was then added and aliquots of the reaction were terminated at the indicated times by the addition of 20 mM EDTA and 0.5% SDS. DNA products were resolved in alkaline agarose gels and analyzed by phosphorimager.

Pol II replication of a primer-template (Figure 3b, right)

1.2 nM primed M13mp18 ssDNA was mixed with 4 nM γ-complex, 15 nM β, 50 μM dNTPs, 2 mM ATP, 3 μCi ^{32}P-α-dATP and 1.68 μM SSB for 2 min at 37° C in buffer A. 4 nM wild-type or exonuclease deficient pol II was then added and aliquots of the reaction were terminated at the indicated times by the addition of 20 mM EDTA and 0.5% SDS. DNA products were resolved in alkaline agarose gels and analyzed by phosphorimager.

Replisome replication assay (Fig. 3j)

Reactions containing 100 fmol 5'-biotinylated 100mer rolling circle DNA, 60 μM dCTP and dGTP, 50 μM γ-S-ATP, 4 pmol DnaB[6], 2.5 pmol β_2, 0.5 pmol pol III* (pol III WT or pol III exonuclease minus containing an ε mutant (D12A and E14A) mixed with τ-complex) of a

total volume of 25 μl of buffer B (20 mM TrisHCl (pH 7.5), 0.5 mM EDTA, 5 mM DTT, 10% glycerol, 8 mM MgCl) were incubated for 5 min at 37°C then immobilization to streptavidin beads (Invitrogen). Beads were washed three times in 500 μl buffer B containing 12 pmol β_2 and 50 μM γ-S-ATP, 60 μM dCTP, 60 μM dGTP. Replication was initiated by adding 2.5 pmol β_2, 0.5 mM ATP, 480 nM SSB_4, 60 μM dATP, 10 μM dTTP and 3 μCi α^{32}PdTTP in a total volume of 25 μl of buffer B. Reactions were terminated at the indicated times by addition of 25 μl of stop buffer (40 mM HEPES (pH 7.5), 40 mM EDTA, 6% SDS). DNA was released from beads by incubating at 95 °C for 3 min then resolved in 0.8% denaturing alkaline agarose gels.

Replisome recombination-directed replication (Supplementary Fig. 3)

20 μM (in nucleotides) RP192 ssDNA was incubated with 6.5 μM RecA, 0.5 mM ATP, 40 mM phosphocreatine, 1 μg creatine phosphokinase, and 250 μM dNTPs in a total volume of 5 μl of buffer B (25 mM TrisHCl pH 7.5, 0.1 mg/ml BSA, 10 mM DTT, 15 mM MgCl) for 5 min. 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 ^{32}P-α-dATP and 7 μM SSB for a further 1.5 min. Next, 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/B/C, 612 nM DnaB, 714 nM DnaC, 680 nM DnaG, 306 nM DnaT, 25.5 nM 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 non-denaturing agarose gels (and in an alkaline agarose gel where indicated) and analyzed by phosphorimager. Reactions were performed at 37° C.

Pol III replication of a primer-template (Fig. 3i)

Reactions contained 37.5 fmol primed M13mp18 ssDNA, 60 μM dCTP and dGTP, 0.5 mM ATP, 0.9 μg SSB, 350 fmol β_2 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-HC1 (pH 7.5), 4% glycerol, 0.1 mM EDTA, 40 μg/ml BSA, 5 mM DTT, 10 mM $MgOAc_2$). Reactions were incubated at 37° C for 5 min, before adding 60 μM dATP, 20 μM dTTP and 1 μCi α^{32}P-dTTP were added to initiate replication. Total reaction volume was 25 μl. DNA synthesis was quenched after the indicated time points by adding 25 μl of 40 mM EDTA and 1% SDS. Quenched reactions were resolved in an alkaline agarose gel and analyzed by phosphorimager.

Exonuclease activity assay (Fig. 3k)

10 nM ^{32}P-labeled primer-template (25/10 or 25G/10 (contains mismatch)) was mixed with 8 mM MgCl, 200 nM β, 60 nM τ-complex, 1 mM ATP and 1 μM SSB in buffer C (20 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 5 mM DTT, 10% glycerol) for 2 min. 30 nM pol II or pol III was added as indicated 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 Exo-), pol IV, pol V, β, and reconstituted γ-complex were purified as described[23,45,46]. τ-complex was reconstituted from pure proteins and purified in an identical fashion as γ-complex, however, γ was replaced with τ His-tagged versions of PriA/B/C and DnaT were purified by standard Ni^{2+} affinity chromatography methods. DnaG/B/C were purified as described [46,47]. Wild-type and exonuclease deficient pol II were purified as described[37,48]. RuvA/B were purified as described[49]. RecA, Pol I (Klenow fragment and Klenow fragment 3'–5' exonuclease minus) and Gyrase were purchased from New England Biolabs.

DNA

Supercoiled plasmid DNA (pRP27) was purified using a Qiagen Maxi-prep kit then centrifuged through a cesium chloride density gradient. 100mer rolling circle DNA was purified as described[50]. Primed M13mp18 ssDNA was prepared as described[45]. Linear double-strand DNA template was prepared as described[51]. Oligonucleotides (5'–3'): RP192, TTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGATCTGCAGTAATACG ACTCACTATAGG GAGGAGGGAGGGATGAGAGAATATTGGG; RP158, GGTACGCGATAATCAGCTGAGACCGCAATACGGATAAGGGCTGAGCACGTCCT GCGATCTGCAGC CTGCCAGAATCTGTG; RP219A, AAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGC CCTTTCGTCTTC AAGAAT; 25, CACAGATTCTGGCAGGCTGCAGATCGC; 10, AGCTGAGACCGCAATACGGATAAGGGCTGAGCACGTCCTGCGATCTGCAGCCTG CCAGAATCTGT G; 25G, CACAGATTCTGGCAGGCTGCAGATCGG; 312, TCACGAGGCCCTTTCGTCTTCAAGAAT; 313, TGTCAAACATGAGAATTCTTGAAGACGAAAGGGCCTCGTGA; 235A, TTTGACAGCTTATCATCGATCTGCAGTAATACGACTCACTATAGGGAGGAGGGA GGGATGAGAGA ATAT; RP235AM1, TTTGACAGCTTATCATCGATCTGCAGTAATACGACTCACTATAGGGAGGAGGGA GGGATGAGAGA ATATGGGA; 235AM2, TTTGACAGCTTATCATCGATCTGCAGTAATACGACTCACTATAGGGAGGAGGGA GGGATGAGAGA ATATGGGGA

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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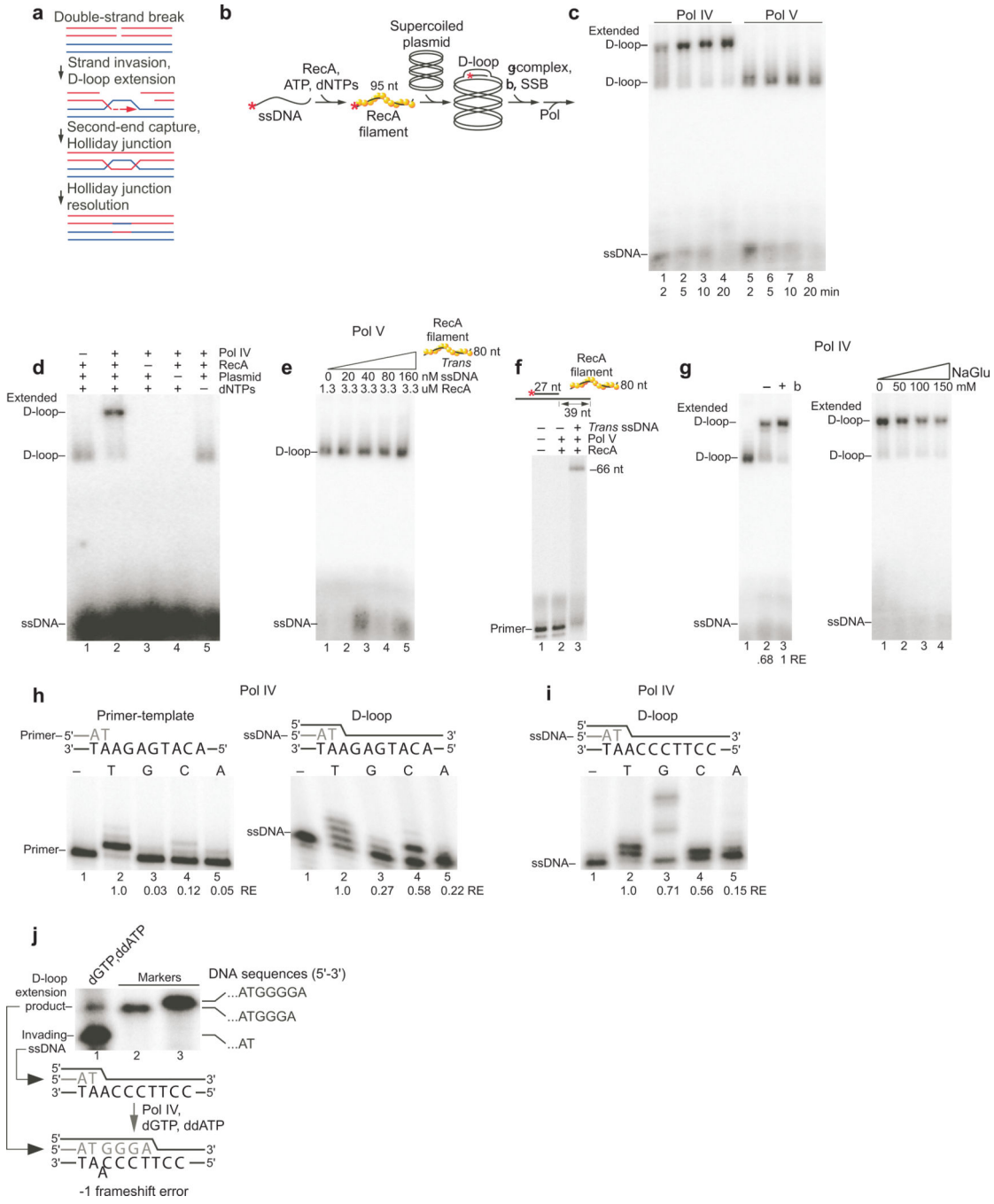

Fig. 1. Pol IV is highly proficient and error-prone in recombination-directed replication

(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 then Holliday junctions are formed which are subsequently resolved by an endonuclease. **(b)** Scheme for reconstitution of RDR (D-loop extension). A 5'-^{32}P labeled ssDNA is incubated with RecA, ATP and dNTPs which promotes RecA filament formation. A supercoiled plasmid containing the same sequence as the ssDNA is then added which facilitates D-loop formation. The β-clamp, which confers processivity

onto pols, is then assembled at the D-loop by adding β along with its clamp-loader (γ-complex) and SSB. Last, DNA polymerase is added which initiates RDR by extending the D-loop. **(c)** RDR was performed with 500 nM pol IV (lanes 1–4) or pol V (lanes 5–8) for the indicated times. **(d)** Controls for pol IV RDR activity. RDR was performed as in (c) in the presence or absence of the indicated reagents. **(e)** RDR was performed with 500 nM pol V in the presence of increasing amounts of ssDNA and 3.3 μM RecA. **(f)** Primer extension was performed with 500 nM pol V and 2 μM RecA in the presence (lane 3) and absence (lane 2) of 160 nM *trans* ssDNA. * indicates ^{32}P. **(g)** RDR was performed with pol IV at relative concentrations corresponding to SOS-induced cells in the presence (lane 3, left) and absence (lane 2, left; right) of β with (right) or without (left) increasing amounts of sodium glutamate (NaGlu). Relative D-loop extension (RE) was determined by dividing the fraction of D-loop extension observed in lane 2 by that observed in lane 3 (left). Fraction of D-loop extension was determined by dividing the intensity of the extended D-loop product by the sum of the intensities of the unextended and extended D-loop products. **(h)** Primer (left) and D-loop (right) extension were performed with pol IV and the indicated dNTP. D-loops were purified and DNA products were resolved in denaturing urea polyacrylamide gels. RE was determined by dividing the fraction of extension products for each lane by the fraction of extension products in lane 2 for each panel. **(i)** D-loop extension was performed as in (h). **(j)** RDR was performed with pol IV in the presence of 50 μM dGTP and 10 μM 2',3'-dideoxyadenosine triphosphate (ddATP)(lane 1). Incorporation of the ddAMP chain terminator opposite the thymidine base (T) 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 2 which indicates that the D-loop was extended by the incorporation of 3 dGMPs and 1 ddAMP demonstrating a −1 frameshift mutation (see schematic at bottom). Partial DNA sequences of the invading ssDNA and markers are indicated. β-clamp, clamp-loader, and SSB were present in all reactions except where indicated.

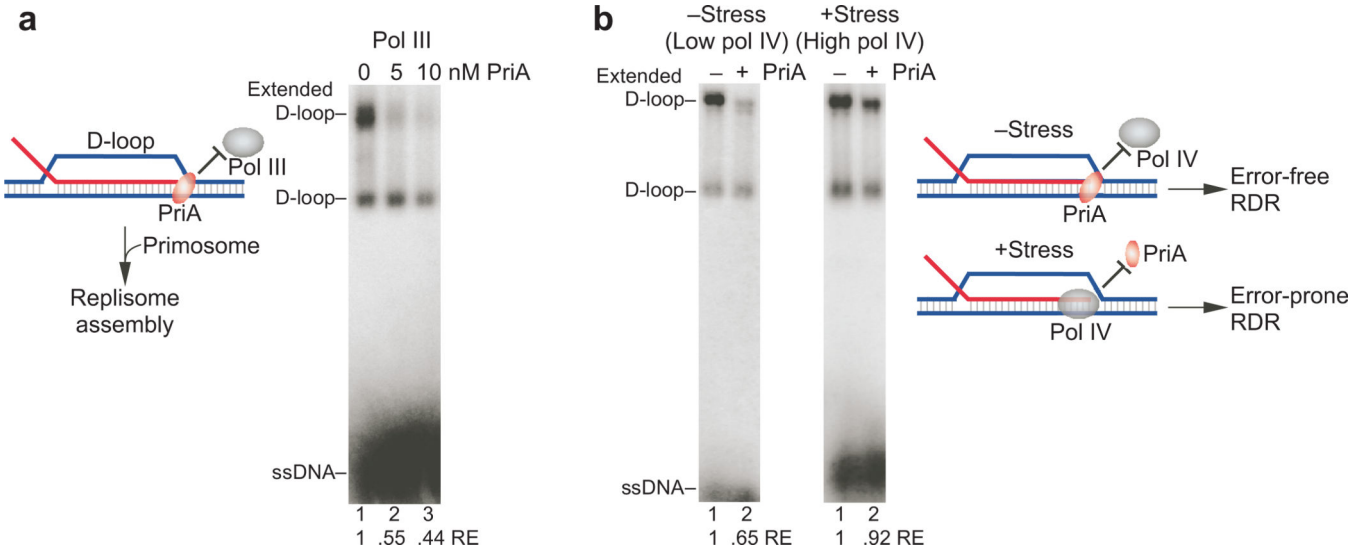

Fig. 2. High levels of pol IV comparable to SOS-induced cells facilitate its recruitment to D-loops

(a) Schematic of PriA inhibition of D-loop extension by pol III (left). RDR was performed with pol III and the indicated concentrations of PriA (right). **(b)** RDR was performed with pol IV concentrations corresponding to non-SOS (left panel) and SOS (right panel) conditions in the presence (lanes 2) and absence (lanes 1) of relative cellular levels of PriA (175 nM). Models of competition between pol IV and PriA at D-loops during non-stressed and stressed conditions (right). Relative D-loop extension (RE) was determined as in Fig. 1. β-clamp, clamp-loader, and SSB were present in all reactions.

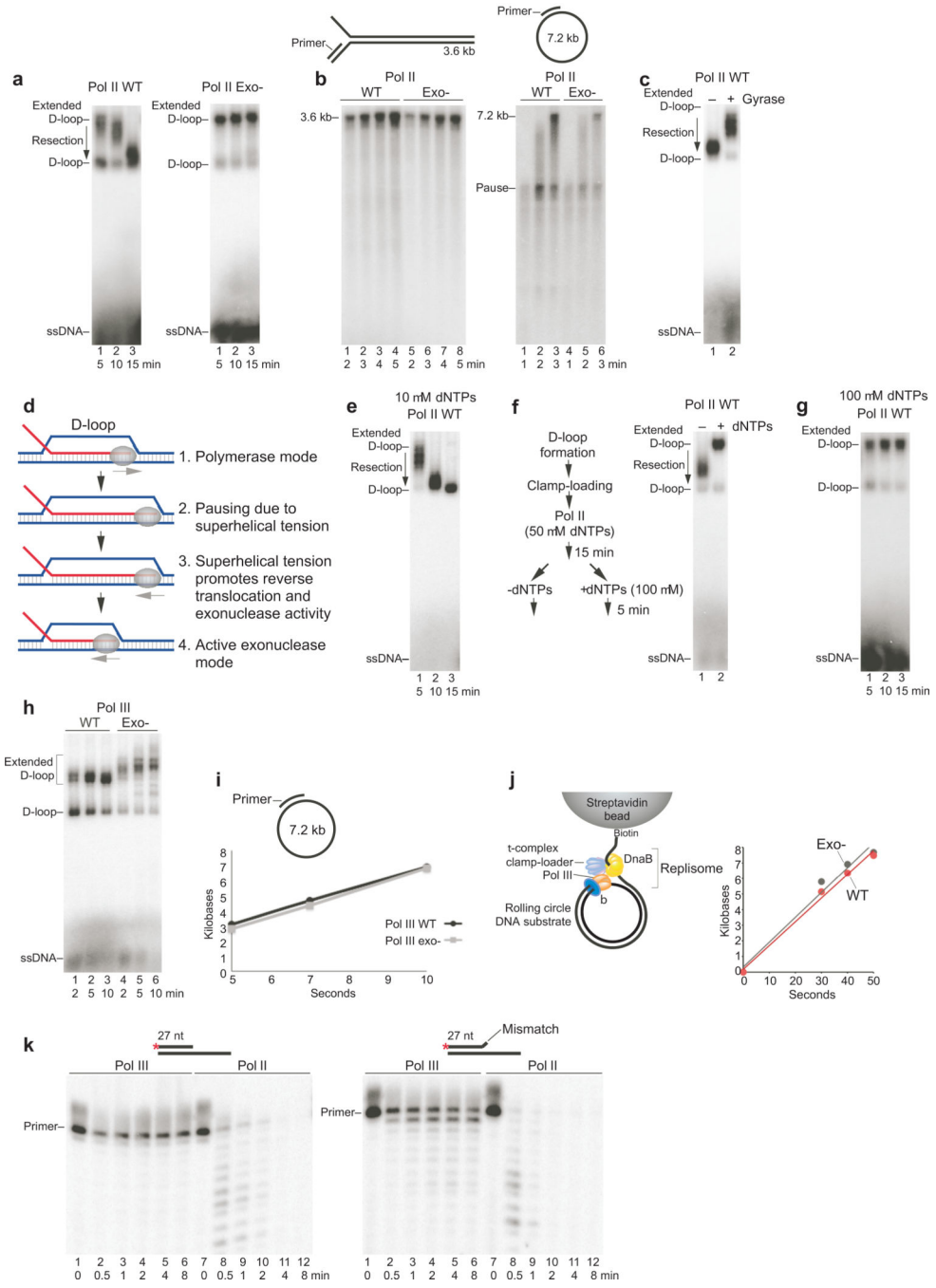

Fig. 3. Pol II switches to an active exonuclease mode at D-loops

(a) A timecourse of RDR was performed with wild-type (left) and exonuclease deficient (right) pol II. **(b)** A timecourse of replication was performed by wild-type and exonuclease deficient pol II on a linear double-strand DNA template (left) and a circular primer-template (right). **(c)** RDR was performed with wild-type pol II in the presence (lane 2) and absence (lane 1) of gyrase. **(d)** Model of pol II activity at D-loops. 1. Pol II engages its polymerase mode to extend a D-loop. 2. Pol II pauses due to superhelical tension in the DNA. 3. Superhelical tension in the DNA promotes reverse translocation and exonuclease activity of

pol II. 4. Pol II switches to a highly active exonuclease mode. **(e)** A timecourse of RDR was performed with wild-type pol II and 10 μM dNTPs. **(f)** RDR was performed with pol II and 50 μM dNTPs for 15 min, the reaction was then divided and aliquots were incubated for a further 5 min in the presence (lane 2) or absence (lane 1) of 100 μM dNTPs. **(g)** A timecourse of RDR was performed with wild-type pol II and 100 μM dNTPs. **(h)** A timecourse of RDR was performed with wild-type (lanes 1–3) or exonuclease deficient (lanes 4–6) pol III and 50 μM dNTPs. **(i)** A timecourse of replication by wild-type (black) and exonuclease deficient (grey) pol III holoenzyme (pol III, β) was performed with 50 μM dNTPs on a m13 primer-template substrate. DNA products were analyzed in a denaturing alkaline agarose gel and analyzed by phosphorimager. **(j)** The replisome containing DnaB, β, τ-complex and either wild-type (red circles) or exonuclease deficient (grey circles) pol III was assembled on a rolling circle template immobilized to streptavidin beads in the presence of dGTP and dCTP. Unbound proteins except for β were removed by washing then a timecourse of leading strand synthesis was initiated by adding dATP, ^{32}P-α-dTTP and SSB. DNA products were analyzed as in (i). **(k)** Pol III (lanes 2–6) or pol II (lanes 8–12) was incubated with a radio-labeled primer-template with (right) or without (left) a mismatch in the absence of dNTPs for the indicated times. DNA products were resolved in a denaturing gel. β-clamp, clamp-loader, and SSB were present in all reactions.

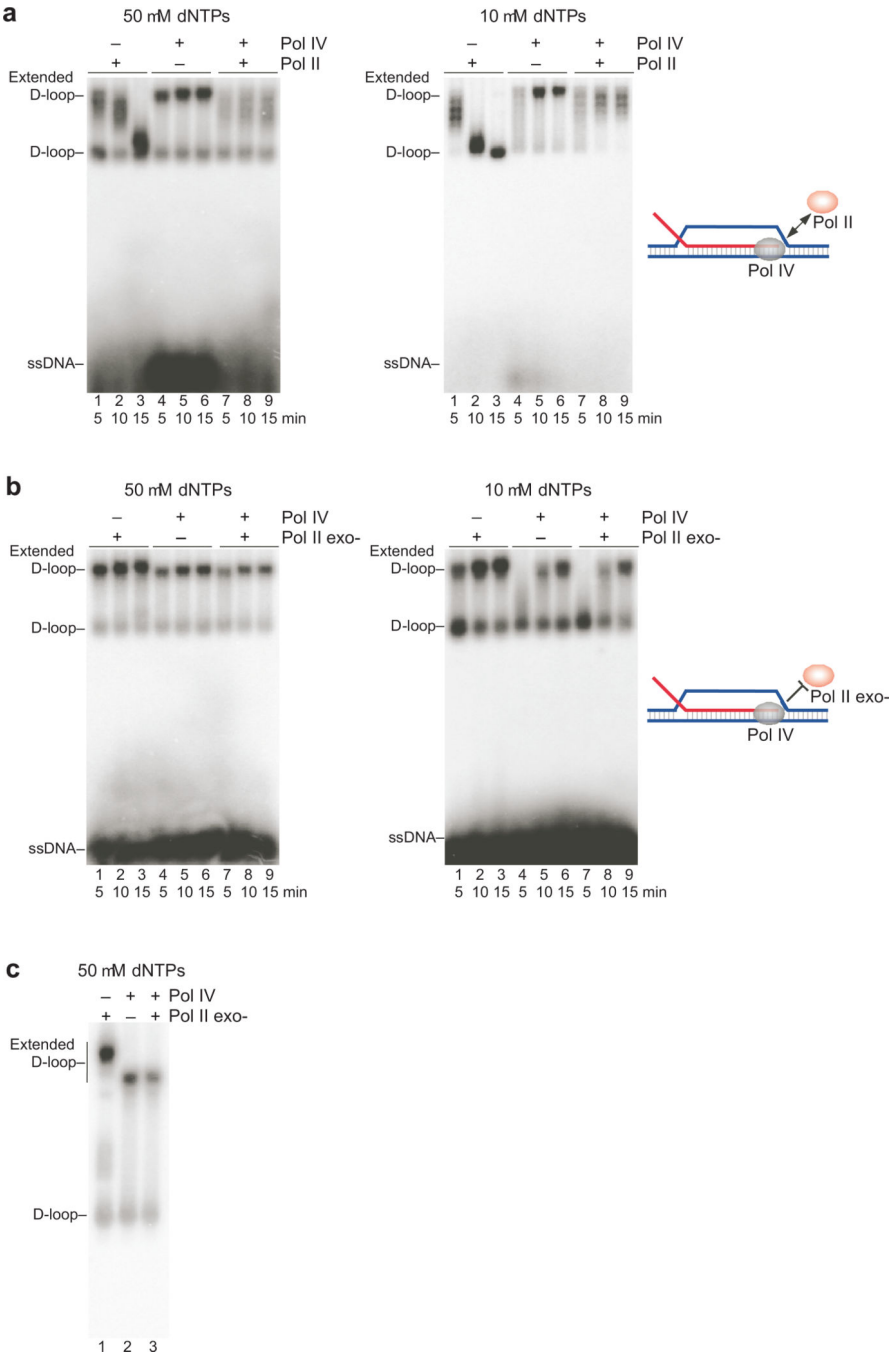

Fig. 4. Pol II requires a functional exonuclease domain to compete with pol IV at D-loops

(a) A timecourse of RDR was performed with pol II (lanes 1–3), pol IV (lanes 4–6), and pol II and pol IV together (lanes 7–9) at relative concentrations corresponding to SOS-induced cells with 50 μM (left panel) or 10 μM (right panel) dNTPs. Schematic representation of results illustrates competition between pol II and pol IV at D-loops (right). **(b)** A timecourse of RDR was performed with exonuclease deficient pol II (lanes 1–3), pol IV (lanes 4–6), and exonuclease deficient pol II and pol IV together (lanes 7–9) at relative concentrations corresponding to SOS-induced cells with 50 μM (left) or 10 μM (right) dNTPs. Schematic

representation of results illustrates the inability of exonuclease deficient pol II to compete with pol IV at D-loops (right). **(c)** RDR was performed with exonuclease deficient pol II (lane 1), pol IV (lane 2), and exonuclease deficient pol II and pol IV together (lane 3) at relative concentrations corresponding to SOS-induced cells with 50 μM dNTPs. β-clamp, clamp-loader, and SSB were present in all reactions.

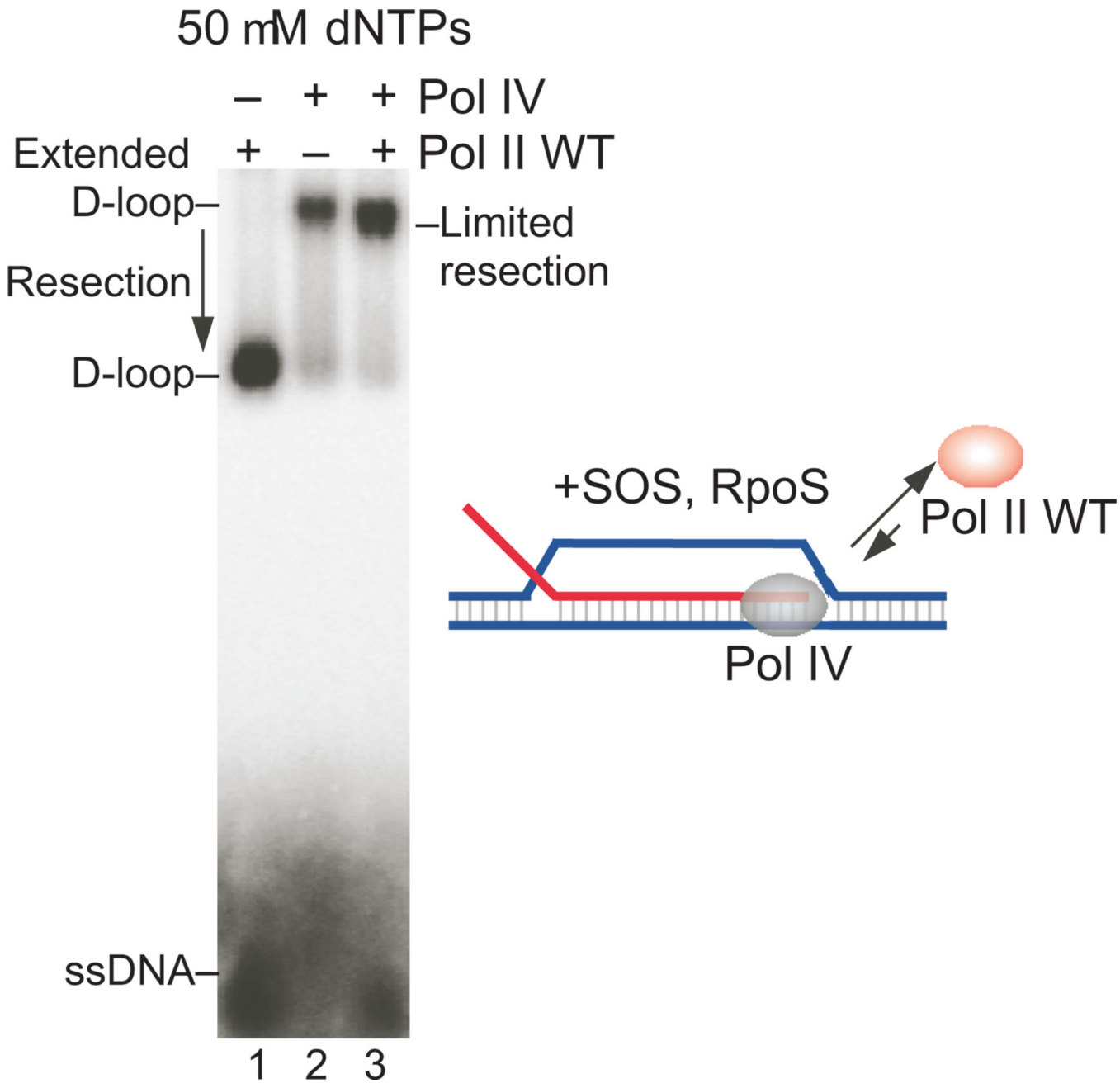

Fig. 5. Levels of pol IV corresponding to RpoS-induced cells allow it to limit pol II exonuclease activity at D-loops

RDR was performed for 30 min with 437.5 nM pol II (lane 1), 6.25 μM of pol IV (lane 2), and pol II and pol IV together (lane 3). Schematic representation of results illustrates limited activity of pol II at D-loops (right). β-clamp, clamp-loader, and SSB were present in all reactions.

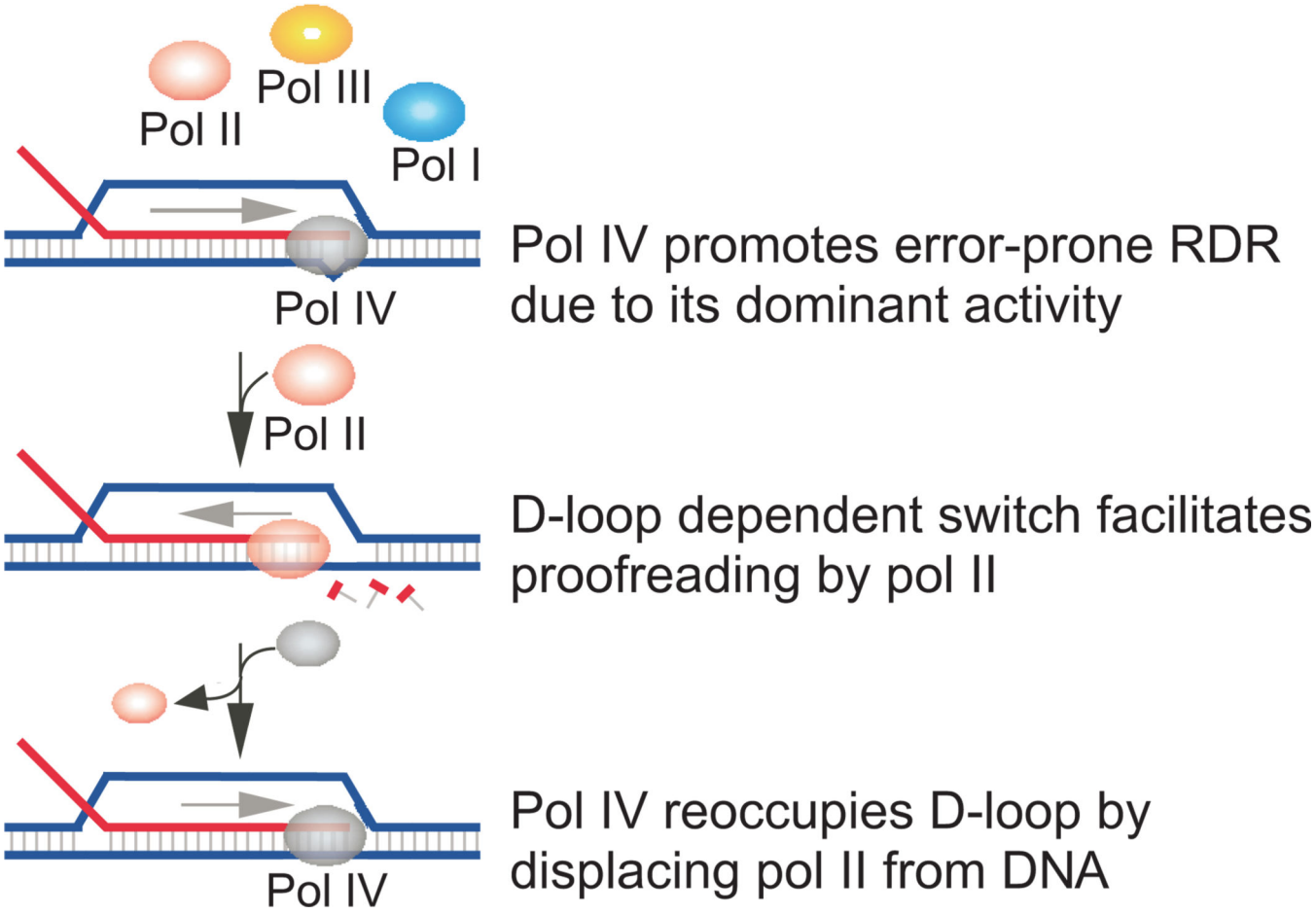

Fig. 6. Model of translesion DNA polymerase activity at D-loops during stress

Upregulation of pol IV by the SOS and RpoS stress responses enables it to outcompete other pols and play a dominant role in RDR during stress which facilitates error-prone recombination. Pol II, however, intermittently competes with pol IV through its exonuclease domain. D-loop dependent stimulation of pol II exonuclease activity enables the polymerase to move in reverse and partially resect the extended D-loop. This activity likely contributes to proofreading of pol IV errors and suppresses error-prone recombination. Pol IV finally regains access to the D-loop by displacing pol II from the DNA.

Table 1

Relative amounts of translesion pols in SOS-induced cells

Pol	+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

mol=molecules

^Based on four chromosomes per cell,

*Two DNA ends estimatec per chromosome due to a single DS3.