Archaeal replicative primases can perform translesion DNA synthesis

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DNA replicases routinely stall at lesions encountered on the template strand, and translesion DNA synthesis (TLS) is used to rescue progression of stalled replisomes. This process requires specialized polymerases that perform translesion DNA synthesis. Although prokaryotes and eukaryotes possess canonical TLS polymerases (Y-family Pols) capable of traversing blocking DNA lesions, most archaea lack these enzymes. Here, we report that archaeal replicative primases (Pri S, primase small subunit) can also perform TLS. Archaeal Pri S can bypass common oxidative DNA lesions, such as 8-Oxo-2′-deoxyguanosines and UV light-induced DNA damage, faithfully bypassing cyclobutane pyrimidine dimers. Although it is well documented that archaeal replicases specifically arrest at deoxyuracils (dUs) due to recognition and binding to the lesions, a replication restart mechanism has not been identified. Here, we report that Pri S efficiently replicates past dUs, even in the presence of stalled replicase complexes, thus providing a mechanism for maintaining replication bypass of these DNA lesions. Together, these findings establish that some replicative primases, previously considered to be solely involved in priming replication, are also TLS proficient and therefore may play important roles in damage tolerance at replication forks.

archaea | replication | translesion synthesis | AEP | primase

The DNA replication machinery rapidly and accurately copies genomes but is prone to stalling at lesions and physical barriers (1). A variety of cellular pathways have evolved to restart stalled replication forks. These include translesion DNA synthesis (TLS) that is performed by specialized polymerases that synthesize short tracts of DNA opposite lesions, thus enabling reinitiation of replication (2). Error-free bypass mechanisms, mediated by homologous recombination, use an alternative undamaged template to rescue stalled replication forks (3). Stalled replisomes can also be rescued by repriming downstream of the blockage, leaving a gap opposite the lesion (4, 5).

Eukaryotes and prokaryotes encode distinct TLS polymerases required for DNA damage tolerance (e.g., Y-family Pols). Although much of our understanding of TLS mechanisms has come from studies of archaeal Y-family DNA polymerases, the majority of archaeal species lack canonical TLS enzymes (Fig. 1*A*) (6), surprising given the otherwise high degree of conservation between eukaryotic and archaeal replisomes. Many archaea do not appear to encode nucleotide excision repair or photolyase pathways that remove UV light-induced damage (6). These anomalies pose the question as to how archaea, lacking canonical TLS or lesion repair pathways, tolerate the presence of lesions that stall replication. This is particularly pertinent to archaea because of the harsh environmental conditions under which many species reside, including extreme temperatures, which promote increased levels of DNA damage.

Archaeal replicases (B- and D-family Pols) specifically arrest at deoxyuracil (dU) (7, 8). This unique feature is limited to replicases from archaea (9). Two important questions regarding dU-induced stalling of archaeal replisomes remain unanswered. First, why do archaea stall replication in response to the template strand dU? Second, how are archaeal genomes containing dU copied? This stalling mechanism may have evolved to prevent promutagenic bypass of the template strand dU, resulting in C–T transition (7, 9). The mechanism used by archaea to resume replication after dU-induced replisome stalling has not been identified.

In this study, we report that archaeal replicative primases (primase small subunit, Pri S) can perform translesion DNA synthesis on damaged DNA templates. Pri S can bypass common DNA lesions, such as oxidative and UV damages, faithfully bypassing cyclobutane pyrimidine dimers (CPDs). Additionally, we report that Pri S can replicate past template strand dUs, even in the presence of stalled replicative polymerase B and proliferating cell nuclear antigen (Pol B/PCNA) complexes, thus providing a specific mechanism for maintaining timely replication of DNA containing dU lesions. Together, these findings establish that the archaeal primase is not only required for de novo primer synthesis during initiation of DNA replication but also actively participates during the elongation step by assisting the major DNA replicases in traversing DNA lesions.

Results and Discussion

Archaeal Primases Replicate Past 8-Oxo-2′-Deoxyguanosines. To address which enzymes are responsible for lesion bypass synthesis in archaea, we first considered which polymerases are present in all archaeal species that could facilitate tolerance of commonly occurring replicase-stalling lesions. One candidate is the replicative primase, a specialized DNA polymerase involved in de novo primer synthesis. In archaea and eukaryotes, the small catalytic subunit primase (Pri S) a member of the archaeo-eukaryotic primase (AEP) family (10), together with the large

Significance

DNA replicases stall at lesions during replication, potentially leading to genome instability. However, cells use specialized lesion bypass polymerases to restart stalled replisomes. Although most organisms possess these damage tolerance polymerases, capable of traversing blocking DNA lesions, many appear to lack these enzymes. We have discovered that replicative primases from archaea, previously considered to be solely involved in priming replication, are also capable of performing translesion DNA synthesis. This discovery has major implications for our understanding of additional roles of DNA primases during replication and the subsequent evolution of related lesion bypass pathways in eukaryotic organisms.

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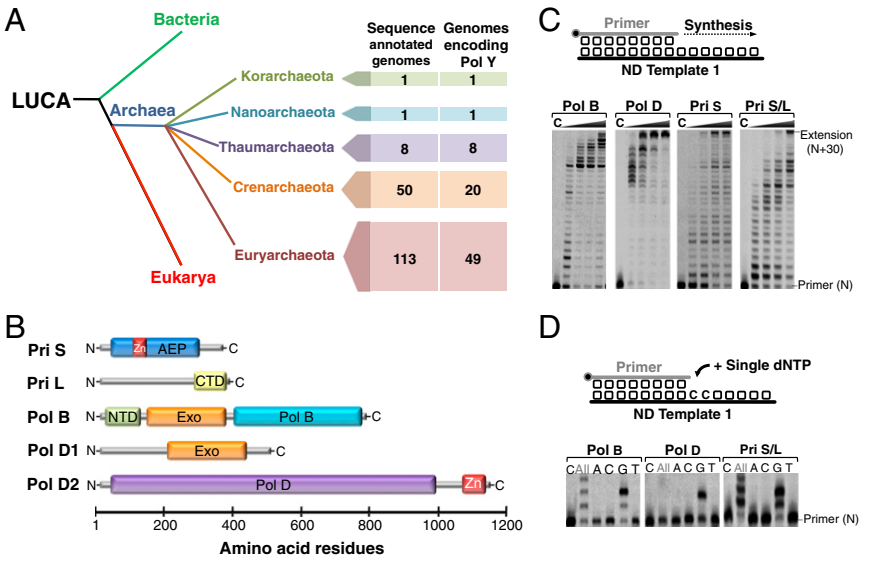

Fig. 1. *A. fulgidus* replisomal enzymes displaying DNA polymerase activity. Analysis of 173 archaeal genomes revealed that only 79 archaea encode canonical TLS DNA polymerases. DNA polymerization activities of *A. fulgidus* replisomal enzymes. (*A*) The absence of genes encoding Y-family DNA polymerases in most archaea is shown. (*B*) Structural elements present in *A. fulgidus* replisomal enzymes. Abbreviations: AEP, archaeo-eukaryotic primase; CTD, carboxy terminal domain; Exo, exonuclease; NTD, amino terminal domain; Pol, polymerase; and Zn, zinc binding site. (*C*) Polymerization on nondamaged templates. (*D*) Single nucleotide incorporation on nondamaged templates. The letter C denotes no enzyme control. The triangles above gel panels indicate time course of the polymerization (30 s, 1′, 5′, and 10′).

subunit (Pri L), is requisite for the initiation of DNA replication (11). Although Pri S has been considered to function exclusively in RNA primer synthesis during replication, archaeal Pri S and related plasmid-borne primases possess both primase and polymerase activities (12–14). In contrast to eukaryotic primases, archaeal Pri S possesses robust DNA polymerase activity (12). It also has terminal transferase activity, suggesting less stringent recognition of DNA substrates (14, 15) and potential involvement in DNA repair processes (16). Notably, closely related prokaryotic and archaeal AEPs, primase-polymerase domain of ligase D (PolDom/LigD) are polymerases involved in the repair of DNA breaks and possess a variety of DNA polymerization activities, including TLS (17, 18). PrimPol, a novel eukaryotic primase, was recently shown to assist in the bypass of lesions encountered during replication (19). Together, these enzymes belong to a growing class of primase polymerases known as PrimPols to reflect their enzymatic activities and origins.

To examine the potential TLS activities of archaeal primases, we cloned, expressed, and purified the catalytically active small primase subunit (Afu-Pri S), heterodimeric primase (Afu-Pri S/L), and the major replicases (Afu-Pol B and Pol D) from *Archaeoglobus fulgidus* (Afu) and the primase holoenzyme (Pfu-Pri S/L) from *Pyrococcus furiosus* (Pfu) (*SI Appendix*, Fig. S1*A*). The structural features of the Afu enzymes studied here are illustrated in Fig. 1*B*. First, we tested the DNA template-dependent polymerase activity of Afu replicative polymerases (Afu-Pol B and Afu-Pol D), primase subunit (Afu-Pri S), and the heterodimeric primase complex (Afu-Pri S/L). All enzymes exhibited robust and error-free DNA polymerase activity (Fig. 1 *C* and *D*). Notably, when we assayed for reverse transcriptase activity, this activity was observed for both primases but not the replicative polymerases (*SI Appendix*, Fig. S3*C*). Again, this suggests that archaeal primases have relaxed substrate specificity, a feature characteristic of TLS polymerases.

The most frequent type of DNA damage is induced by oxidative stress, resulting in the formation of 8-oxo-2′-deoxyguanosine (8-oxo dG) (20). Most replicative DNA polymerases misrecognize 8-oxo dG, resulting in incorrect deoxyadenosine (dA) incorporation opposite to this lesion (21). We tested whether Afu polymerases and primases were promutagenic while traversing 8-oxo dG and observed that all of these enzymes could bypass 8-oxo dG, showing marked stalling before and after the lesion (Fig. 2*A*). A similar profile of bypass past 8-oxo dG was observed for Pfu-Pri S/L (*SI Appendix*, Fig. S1*B*). Next, we investigated the fidelity of 8-oxo dG bypass by Afu and Pfu enzymes using single nucleotide incorporation assays. Family-B DNA polymerase (Afu-Pol B) displayed error-prone bypass of 8-oxo dG, incorporating dA opposite the lesion (Fig. 2*B*). Notably, Afu-Pol D and both primases (Afu-Pri S/L and Pfu-Pri S/L) incorporated the correct deoxycytosine (dC) and incorrect deoxyadenosine (dA) opposite the damage with comparable efficiency (Fig. 2*B* and *SI Appendix*, Fig. S1*C*). Thermophilic archaea are subjected to increased levels of oxidative stress, promoting depurination of 8-oxo dG to abasic site (Ab) and oxidation of thymine to thymine glycol (Tg). However, all of the tested enzymes were strongly blocked by Ab or Tg lesions (*SI Appendix*, Fig. S3 *A* and *B*, respectively), in common with PrimPol (19).

Archaeal Primases Replicate DNA Templates Containing Deoxyuracils. Hydrolytic deamination of deoxycytosine (dC) to dU frequently occurs in DNA (20) and is greatly accelerated by temperature. Therefore, thermophiles are at increased risk from this type of damage (22). Archaeal replicative polymerases (Pol B and D) evolved specifically to avoid replicating past dU and Pol B possesses a uracil-binding pocket (Fig. 1*B*) that scans the template for this lesion in advance of the replicase (23). When detected, the deaminated base is bound tightly and replication arrests four bases before dU (23). Notably, Pol B/PCNA binds dU tightly enough to prevent the lesion being removed by base excision repair enzymes (24). This suggests that the uracil-binding pocket may be important for protecting the integrity of dU-containing DNA during replication. DNA synthesis by Pol D is also markedly inhibited by dU on the template strand (8). To determine if dU inhibition was also evident in *Afu* replicases, we measured whether Afu-Pol B and D could traverse dU and observed that they both profoundly arrested at this lesion (Fig. 2*C*). Next, we assayed for synthesis opposite dU by Afu-Pri S and Afu-Pri S/L and observed that the primase readily bypassed dU (Fig. 2*C*).

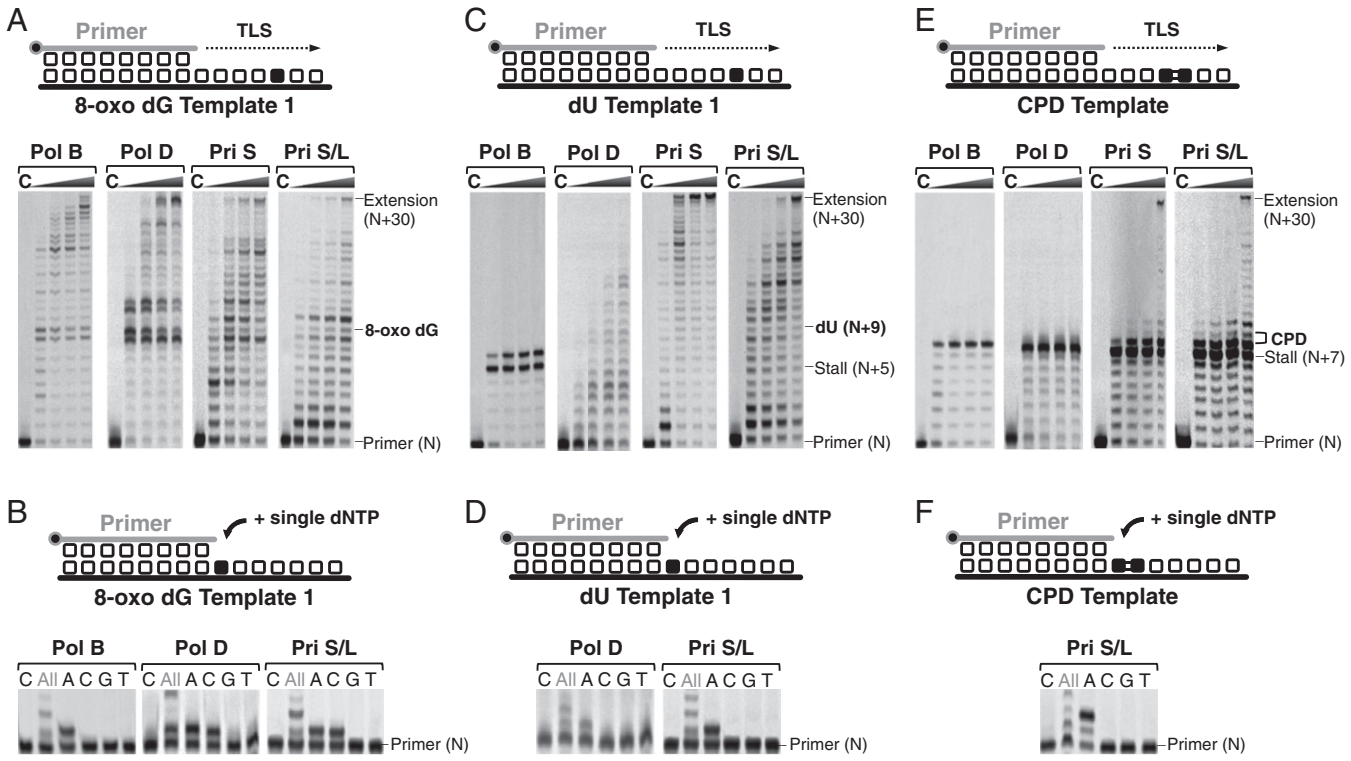

Fig. 2. Translesion synthesis past 8-oxo-dGs, dUs, and CPDs. (*A*) TLS performed on templates containing 8-oxo-dGs. (*B*) Single nucleotide incorporation opposite 8-oxo-dG. (*C*) TLS performed on templates containing dUs. (*D*) Single nucleotide incorporation opposite dU. (*E*) TLS performed on templates containing CPDs. (*F*) Single nucleotide incorporation opposite CPD. The letter C denotes no enzyme control. The triangles above gel panels indicate time course of the polymerization (30 s, 1′, 5′, and 10′).

Bypass of dUs was also observed for Pfu-Pri S/L (*SI Appendix*, Fig. S1*B*). Next, we measured the fidelity of the dU bypass using single nucleotide incorporation assay opposite the lesion. Archaeal primases, specifically incorporated dA opposite dU, indicating that bypass synthesis is promutagenic (Fig. 2*D* and *SI Appendix*, Fig. S1*D*).

Afu Replicative Primase Catalyzes Error-Free Bypass of Cyclobutane Pyrimidine Dimers. Theremophilic archaea, including *Archaeoglobus* and *Pyrococcus*, tolerate high doses of UV light (25, 26), notable given their apparent lack of recognizable lesion repair or bypass mechanisms. UV induces DNA lesions, particularly cross-links between adjacent pyrimidine bases including: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) modifications (27). These UV photoproducts distort DNA and act as potent replication blocking lesions (2, 21). Similar replicase stalling lesions can occur at significant rates in organisms not exposed to light, e.g., produced by cross-linking with aldehyde (28). Cross-linking rates are accelerated by oxidative stress; therefore, this type of DNA damage may be abundant in thermophilic archaea. First, we tested whether Afu-Pri S has TLS activity on templates containing CPDs. Whereas the replicative polymerases were incapable of bypassing CPDs, both Afu-Pri S and Afu-Pri S/L performed TLS across this UV-induced lesion (Fig. 2*C*), establishing that *Afu* replicative primase can also catalyze bypass of the CPDs. Second, we evaluated the fidelity of the CPD bypass, measuring single base incorporations opposite the damage. Afu-Pri S/L incorporated two dAs opposite both the 3′ and 5′ templating thymines of the CPD (Fig. 2*F*), establishing that archaeal primase catalyses error-free TLS past this UV damage. Notably, the bypass fidelity of Afu-Pri S/L mirrors the TLS activity of Pol η, a eukaryotic Y-family polymerase involved in error-free bypass of CPD (2). In contrast, *Pfu* primase (Pfu-Pri S/L) was unable to traverse the CPD, incorporating a single correct incoming base (dA) opposite the first (3′) base of the dimer (*SI Appendix*, Fig. S1 *B* and *E*). Next, we measured the capacity of archaeal enzymes to replicate past the 6-4PPs. The archaeal enzymes were unable to bypass this UV damage (*SI Appendix*, Fig. S3*D*). Although PrimPol can bypass this lesion (19), 6-4PPs cannot be traversed by other TLS DNA polymerases (2, 27). Bypass often requires the collaborative effort of two specialized enzymes, where the first performs insertion opposite the 6-4PP (an "inserter" polymerase) and the second extends the primer bearing 3′ terminal base aligned with the dimer (an "extender" polymerase) (2, 21). We therefore measured if the archaeal enzymes display TLS "extender" abilities on UV lesions and observed that both primases could extend primers containing 3′ terminal bases annealed to the CPDs and 6-4PPs (*SI Appendix*, Figs. S1*G*, S2 *A* and *B*, and S4 *B* and *D*); see *SI Appendix*, *SI Results* for details.

Afu Primase Rescues Pol B Stalled at the Template Strand Deoxyuracil. Although archaeal replicases bind dU with nanomolar affinity (29), a restart mechanism has not yet been identified. To address whether the replicative primase plays a role in restarting arrested replisomes, we assayed TLS activities of Afu-Pri S/L on short templates (30 nt), containing a single dU, preincubated with the Pol B/PCNA complex (Fig. 3*A*). We observed that Pri S/L retained robust TLS bypass activity on dU-containing templates, even in the presence of stalled replisome components. These data indicate that Pri S/L has an innate capacity to access the 3′ end of the primer, even when Pol B/PCNA complex has stalled at dU. This is a notable observation given that Pol B/PCNA stalling at dU abrogates detection and removal of deaminated bases by DNA glycosylases (24).

As Pri S/L assists in the bypass of replication blocking lesions, we next investigated whether archaeal primases function as a

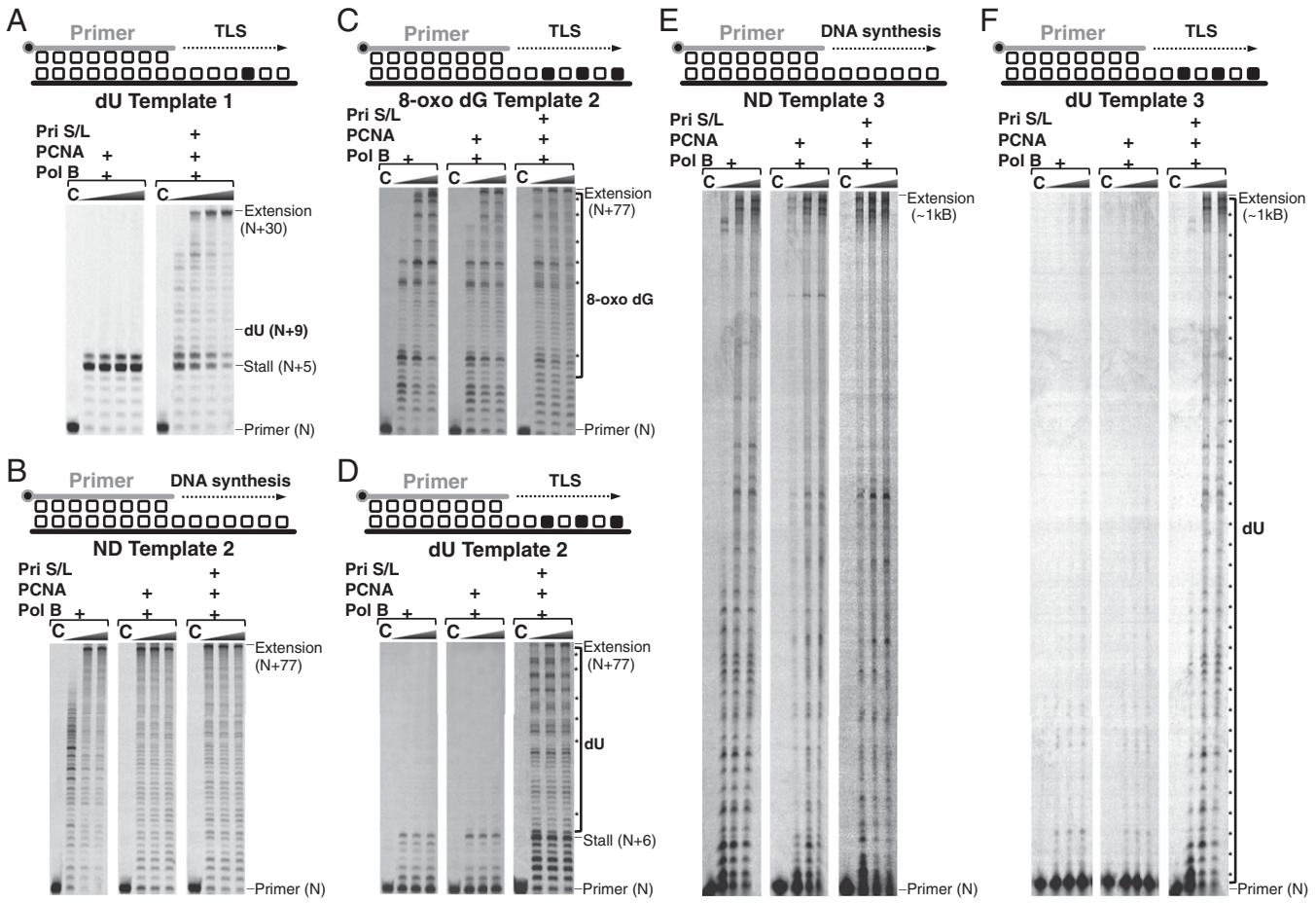

Fig. 3. Translesion synthesis past multiple DNA damage. (*A*) Pol B/PCNA stalls four bases before dU (*Left*). Stalled Pol B/PCNA is rescued when TLS past dU is performed by Pri S/L (*Right*). (*B*) Control primer extension on nondamaged substrates. Polymerization performed by Pol B (*Left*), Pol B/PCNA (*Middle*), and Pol B/PCNA and Pri S/L (*Right*). (*C*) Primer extension on templates containing seven 8-oxo dGs. TLS performed by Pol B and Pol B/PCNA results in full-length product (*Left* and *Middle*, respectively) but pronounced pausing pattern opposite to 8-oxo dG is observed. Polymerization performed by Pol B/PCNA and Pri S/L results in efficient primer extension. (*D*) Polymerization on a DNA template containing seven dUs performed by Pol B and Pol B/PCNA is strongly inhibited (*Left* and *Middle*, respectively). In contrast, DNA synthesis performed by Pol B/PCNA and Pri S/L resulted in bypass of the multiple dUs. (*E*) Primer extension on long nondamaged templates. Polymerization was performed by Pol B (*Left*), Pol B/PCNA (*Middle*), Pol B/PCNA and Pri S/L (*Right*). (*F*) Polymerization on long templates containing ~20 randomly distributed dUs. Strong inhibition of DNA synthesis is observed for Pol B and Pol B/PCNA (*Left* and *Middle*, respectively). Again, DNA synthesis performed by Pol B/PCNA and Pri S/L resulted in full-length primer extension. The letter C denotes no enzyme control. The triangles above gel panels indicate time course of the polymerization; *A* (30 s, 1′, 5′, and 10′), *B* and *C* (1′, 5′, and 10′), and *E* and *F* (3′, 10′, and 20′).

component of the replication machinery, required to maintain progression on templates containing multiple lesions. To address this possibility, we assayed the capacity of Afu-Pol B and Pol B/PCNA to replicate DNA templates (77 nt) containing multiple 8-oxo dG lesions, in the presence or absence of Pri S/L (Fig. 3 *B* and *C*). Although Pol B and Pol B/PCNA fully extended primers on templates containing multiple 8-oxo dG, a regular pausing pattern in close proximity to the lesions was observed, compared with nondamaged template extension (Fig. 3 *B* and *C*, *Left* and *Middle*). This indicates that a significant slowing of the replicase was occurring. Overall, levels of DNA synthesis were enhanced by the addition of PCNA on nondamaged templates but not on templates containing multiple 8-oxo dGs. However, addition of Pri S/L restored rates of primer extension to those observed on undamaged primer templates (Fig. 3 *B* and 3*C*, *Right*).

Next, we examined the capacity of Afu-Pol B to replicate DNA templates containing multiple dUs. In contrast with templates containing multiple 8-oxo dGs, Pol B alone or assisted by PCNA was unable to traverse multiple dUs (Fig. 3*D*, *Left* and *Middle*). Again, addition of Pri S/L restored efficient DNA synthesis on this heavily damaged template (Fig. 3*D*, *Right*), indicating that the archaeal primase and polymerase may collaborate to maintain efficient replication fork progression on DNA containing multiple dU "roadblocks." To address whether the primase facilitates the maintenance of robust and processive DNA replication on much longer DNA templates, a ~1-kb-long DNA template was prepared containing multiple dUs (~20 lesions per template). As anticipated, Afu-Pol B alone or assisted by PCNA displayed highly processive synthesis on the undamaged template (Fig. 3*E*) and profound stalling on dU-containing templates (Fig. 3*F*, *Left* and *Middle*). However, addition of Afu-Pri S/L again rescued DNA synthesis by Afu-Pol B/PCNA complex (Fig. 3*F*, *Right*). Together, these data provide evidence that primases, in addition to their role in initiation of DNA synthesis, also actively participate in the elongation phase of DNA replication by performing TLS bypass of lesions thus preventing the archaeal replisome from arresting (Fig. 4). This interplay between replicase and primase appears to be important for processive synthesis to ensure timely DNA replication.

Concluding Remarks

It has been widely assumed that AEP-like primases evolved as replication enzymes relatively late in evolution, based on their

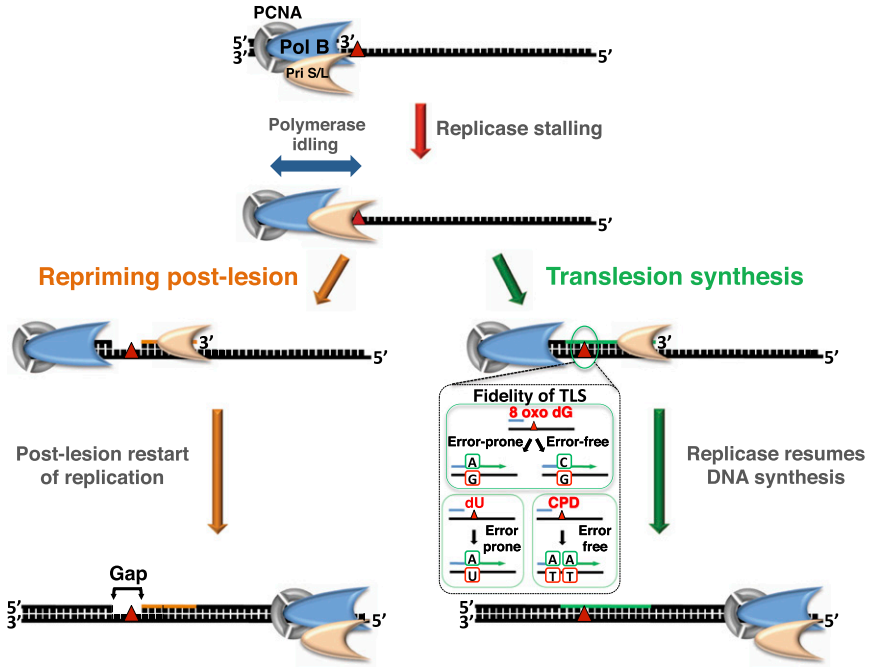

Fig. 4. Collaboration of the core components of the archaeal replisome results in bypass of DNA lesions. *Top* shows polymerizing replicase (Pol B, blue) with sliding clamp (PCNA, gray). The complex encounters blocking DNA lesion (red triangle) resulting in Pol B idling, which allows recruitment of the primase (Pri S/L, yellow). Depending on the type of the lesion, Pri S/L employs either primase or translesion synthesis (TLS) activity. *Left* illustrates a scenario where the blocking lesion is relatively large (i.e., large aromatic organic compound or protein covalently attached to DNA) and Pri S/L synthesizes a short primer (orange) after the damage so that the Pol B/PCNA complex can restart replication downstream from the block. Alternatively, when the blocking lesion is small (e.g., 8-oxo dG, dU, or CPD), TLS (green) is performed by Pri S/L so that the Pol B/PCNA complex resumes DNA synthesis. The fidelity of TLS performed by Afu-Pri S/L is shown.

initial discovery in eukaryotic organisms. However, it is now apparent that both the evolutionary origins and roles of enzymes belonging to the AEP superfamily must be significantly reevaluated. Primordial AEPs first originated in prokaryotes and bacteriophage to perform a diverse range of roles in DNA metabolism, including DNA repair (17, 18). Indeed, many bacterial species possess multiple AEP enzymes, in addition to possession of a replicative DnaG primase, suggesting an early diversification of functions within the AEP superfamily. During evolution of archaea from the last universal common ancestor (LUCA), both DnaG and AEPs were maintained but the essential role of DnaG as the replicative primase was superseded by AEP primases (30). Presumably, these enzymes offered particular advantages to these newly evolving organisms, including the capacity to traverse DNA lesions. Recently, a second AEP (PrimPol) has been identified in higher eukaryotes (10), whose primase and polymerase functions emulate those described here for archaeal replicative primases (19, 31). PrimPol was probably acquired from large cytoplasmic viruses during the early evolution of the eukarya (10), where it superseded Pri S's lesion bypass role during replication. This potentially allowed eukaryotic Pri S to assume more specialized roles in primer synthesis. This demarcation of the primase/polymerase activities between PrimPol and Pri S probably reflects the additional replication requirements for much larger genomes, such as more regulated lagging-strand synthesis.

Although some archaeal species possess canonical Y-family DNA polymerases (Dpo4), specialized in traversing DNA lesions, a recent study has reported that *Sulfolobus* strains lacking Dpo4 display no increased sensitivity to DNA damaging agents, including UV (32). This strain also exhibited no difference in rates of spontaneous mutagenesis, suggesting that other TLS pathways assist in bypassing replication-blocking lesions. Notably in this regard, although expression of the DNA replication genes is down-regulated after exposure of *Sulfulobus* cells to UV, Pri S/L was up-regulated after irradiation, supporting its proposed role in TLS (33). In addition, ethyl methanesulfonate (EMS) and UV treatments of *Pyrococcus* strains, which also lack canonical TLS polymerases, resulted in a strongly induced mutagenesis, with spontaneous mutation frequencies increased ~150-fold after EMS treatment and ~400-fold after UV exposure (26). The observed DNA damage induced mutagenesis indicates the possible existence of an active TLS pathway operating in these organisms. This predication is also supported by fractionation studies of whole cell extracts from *P. furiosus* that identified distinct polymerase activities in three major fractions (34). Notably, one of these fractions contained apparent TLS activity and, although the polymerase responsible for this was not identified, it was fully coincident with the elution of the Pri S/L complex. Together, with the findings presented here, these studies add support to our model postulating that archaeal replicative primases are involved in synthesis past DNA lesions during replication.

Since the discovery of canonical TLS polymerases, it has been widely assumed that these enzymes are largely responsible for TLS during replication (2). However, this study identifies that replicative primases can also act as proficient TLS polymerases that assist replicases in bypassing blocking DNA lesions during replication. As these enzymes are core replisomal factors, it argues that in many organisms the replisome is inherently TLS proficient and other damage tolerance mechanisms may provide additional assistance in a postreplicative manner. This discovery has major implications for our understanding of additional roles of DNA primases during replication and the subsequent evolution of related PrimPol-centric TLS pathways in eukaryotic cells (19, 31). Although this report has focused on the TLS activities of archaeal Pri S/L, these enzymes are also proficient DNA primases and therefore their ability to reprime replication postlesion (Fig. 4),

proposed for other organisms (4, 5, 19, 35), is also likely to be important.

Materials and Methods

Materials. Deoxynucleotide triphosphates (dNTPs) and deoxyuracil triphosphate (dUTP) were from by Roche and Jena Bioscience, respectively. Enzymes were provided by New England Biolabs.

Cloning of Archaeal Genes. *A. fulgidus* and *P. furiosus* genes were PCR amplified and cloned using standard molecular biology techniques. All of the PCR primers (Eurofins MWG Operon) sequences used in this study are listed in *SI Appendix*, Table S1.

Expression and Purification of Archaeal Proteins. Archaeal proteins were produced in *Escherichia coli* Rosetta strain (Novagene) grown at 37 °C. Typical purification procedure is composed of three chromatography steps: immobilized metal affinity, ion exchange, and gel filtration. Afu-PCNA was expressed and purified as previously described (36).

Synthetic Primer Templates. The oligodeoxynucleotides and oligoribonucleotides used to prepare primer templates were purchased from ATDBio and Eurofins MWG Operon, respectively. All primers were fluorescently labeled to aid visualization. Primer templates were annealed by heating equimolar amounts of the oligomers in 10 mM Tris, pH 7.5, 50 mM NaCl, and 0.5 mM EDTA at 95 °C for 5 min, followed by cooling slowly to room temperature. All primer-template sequences used in this study are listed in *SI Appendix*, Table S2.

Enzymatic Preparation of Long Single-Stranded DNA Templates. Approximately 1-kb-long single-stranded DNA (ssDNA) templates were prepared using PCR followed by strand-specific exonucleolytic degradation. Reaction mixtures contained primers listed in *SI Appendix*, Table S1. One of the primers was 5′ end phosphorylated to direct strand-specific degradation. The PCR comprised 50 μL of 1× Taq reaction, 200 μM of the four dNTPs, 1 μM of the forward and reverse primer, 50 ng of pUC18, and 20 units/mL of Taq DNA polymerase. To prepare ssDNA containing dUs, 20 μM of dUTP was added to the PCR mixture. A total of 30 PCR cycles (30 s at 95 °C, 35 s at 55 °C, and 1 min at 72 °C) were used. DNA was resolved and purified on 1% agarose/ethidium bromide gel. PCR product was excised and electroeluted. Recovered dsDNA was ethanol precipitated and the 5′ phosphorylated strand degraded using λ-exonuclease. ssDNA templates were annealed with 20-nt-long primer to yield nondamaged and dU-containing primer templates (*SI Appendix*, Table S3).

Primer Extension and Single Nucleotide Incorporation Assays. Reactions were performed in 20 μL volume containing 20 mM Tris, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_2$, 2 mM $MgSO_4$, and 20 nM primer template. The polymerization was monitored for variety of archaeal enzymes, including 50 nM replicases and 50 nM of primases. Some of the reactions were supplemented with PCNA (200 nM). For running start extensions, 50 μM of each of the four dNTPs was used. In cases of single dNTP addition, 50 μM of the particular dNTP under investigation was added. All reactions were carried out at 50 °C and quenched by addition of an equal volume of 95% (vol/vol) formamide/5% (vol/vol) water containing 20 mM EDTA. Primer extensions were carried out for the time indicated in legends of Figs. 1–3 and *SI Appendix*, Figs. S1–S5. All single nucleotide incorporations were terminated after 5 min. Polymerization products were resolved on 15% (wt/vol) denaturing polyacrylamide gels containing 7 M urea. The gels were visualized using fluorescent scanner Fuji FLA-150.

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