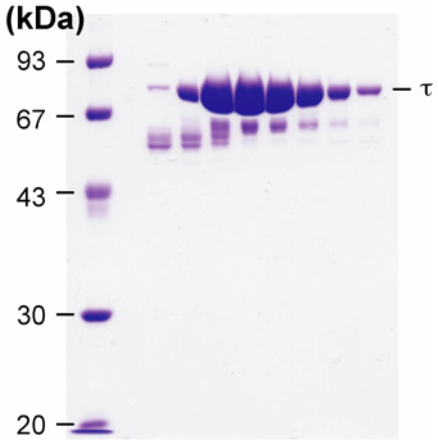
SUPPLEMENTARY MATERIAL:

Single-Molecule Studies of Fork Dynamics in *Escherichia coli* DNA Replication

Tanner, Nathan A.[1,2]; Hamdan, Samir M.[2]; Jergic, Slobodan[3,4]; Schaeffer, Patrick M.[3,5]; Dixon, Nicholas E.[3,4]; & van Oijen, Antoine M.[2,6]

1: Graduate Program in Biological and Biomedical Sciences
2: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Ave. Boston MA USA 02115
3: Research School of Chemistry, Australian National University, Canberra ACT 0200, AUS
4: School of Chemistry, Northfields Ave., University of Wollongong NSW 2522, AUS
5: School of Pharmacy and Molecular Sciences, James Cook University, Townsville QLD 4811, AUS
6: Corresponding Author: antoine_van_oijen@hms.harvard.edu

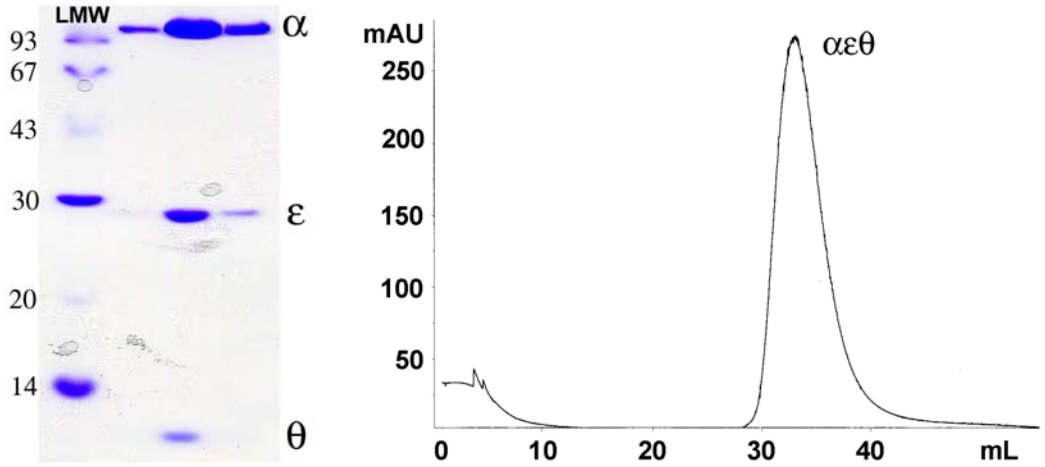
Supplementary Figure 1: Overproduction and purification of the τ subunit of *E. coli* DNA polymerase III



SDS-PAGE of successive fractions from the heparin-Sepharose column (central four fractions comprise Fraction V, below). Procedures were based on methods described by Maki & Kornberg[1] with small modifications. Buffers used were**:** lysis buffer (50 mM Tris.HCl pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20 mM spermidine); buffer Aτ (50 mM Tris.HCl pH 7.6, 1 mM EDTA, 2 mM dithiothreitol); buffer Bτ (50 mM Tris.HCl pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20% v/v glycerol, 150 mM NaCl). Plasmid pJC491, which directs overproduction of τ, but not γ, under control of phage λ promoters, has been described elsewhere[2]. The *ompT* strain BL21(λDE3)*recA*[3] was used as host for production of τ to avoid its proteolytic cleavage by the OmpT protease[2,4]. *E. coli* strain BL21(λDE3)*recA*/pJC491 was grown at 30˚C in LB medium supplemented with glucose (3.6 g l^{-1}), thymine (15 mg l^{-1}) and ampicillin (200 mg l^{-1}). Upon growth to A_{595} = 0.7, the temperature was rapidly increased to 42˚C to induce overproduction, and the 1-liter cultures were shaken for a further 3 h, after which they were chilled in ice. Cells were harvested by centrifugation (11,000 × *g*; 5 min), frozen in liquid nitrogen and stored at –70 ˚C. After thawing, cells (5.5 g from 3 liters of culture) were resuspended in lysis buffer (85 ml) and lysed by being passed twice through a French press (12,000 psi). The lysate was clarified by centrifugation (35,000 × *g*; 30 min) to yield the soluble Fraction I. Proteins that were precipitated from Fraction I by addition of solid ammonium sulfate (0.23 g ml^{-1}) and stirring for 60 min, were collected by centrifugation (35,000 × *g*; 30 min) and dissolved in buffer Aτ+150 mM NaCl (40 ml). The solution was dialyzed against three changes of 2 liters of the same buffer, to yield Fraction II. Fraction II (45 ml) was applied at 1 ml min^{-1} to a column (2.5 x 16 cm) of Toyopearl DEAE-650M resin that had been equilibrated in buffer Aτ+150 mM NaCl. Fractions containing proteins that did not bind to the column were pooled and dialyzed against three changes of 2 liters of buffer Aτ+50 mM NaCl. The dialysate (Fraction III, 60 ml) was loaded at 1 ml min^{-1} onto a column (2.5 × 16 cm) of the same resin, now equilibrated in buffer Aτ+50 mM NaCl. After the column had been washed with 100 ml of the same buffer, τ was eluted in

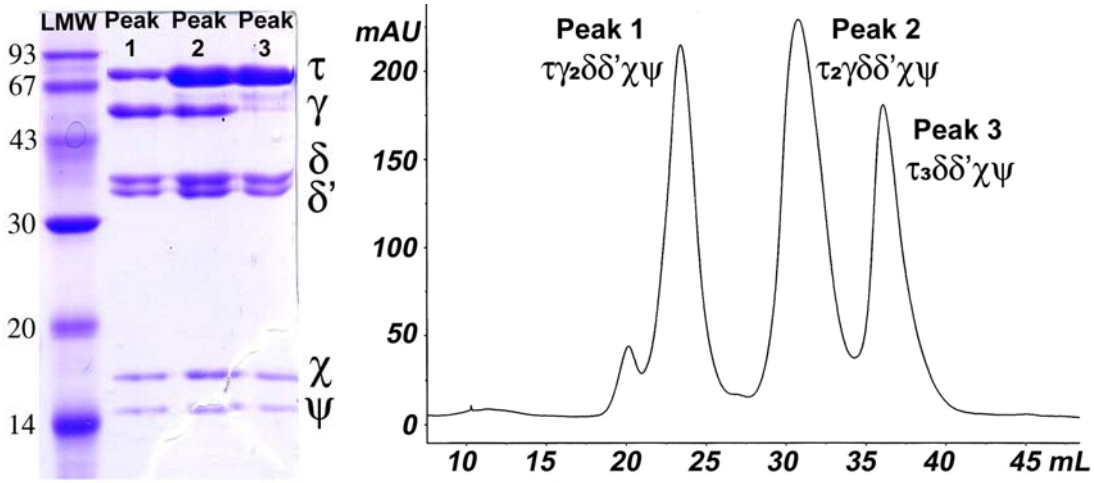
a linear gradient (250 ml) of 50–250 mM NaCl in buffer Aτ. Fractions containing τ were pooled and dialyzed against three changes of 2 liters of buffer Aτ+20 mM NaCl, to yield Fraction IV. Fraction IV (55 ml) was loaded at a flow rate of 0.5 ml min^{-1} onto a column (2.5 × 10 cm) of heparin-Sepharose 4B[5] that had been equilibrated in buffer Aτ+20 mM NaCl. The τ subunit was eluted using a linear gradient (300 ml) of 20–400 mM NaCl in buffer Aτ. It eluted in a single peak at about 220 mM NaCl. Fractions containing τ were pooled and dialyzed against three changes of 2 liters of buffer Bτ to give Fraction V (30 ml containing 93 mg of protein). Aliquots were frozen in liquid nitrogen and stored at −70 ˚C. The molecular weight of τ determined by ESI-MS in 0.1% formic acid (71030±17) may be compared with the calculated value of 71007, and indicated that the N-terminal methionine had been removed. In addition, a modified procedure based on methods used to separate different clamp loader subassemblies[6] was also used. It is similar except that the last purification step was carried out using a column (2.5 × 16 cm) of SP-Sepharose HP (GE Healthcare). Fraction IV (above) was equilibrated in buffer Aτ+50 mM NaCl and loaded at a flow rate of 1 ml min^{-1} onto the SP-Sepharose column that had been equilibrated with the same buffer. The τ subunit was eluted using a linear gradient (700 ml) of 50–450 mM NaCl in buffer Aτ. It eluted in a single sharp peak at about 200 mM NaCl.

Supplementary Figure 2: Separation of the core polymerase (αεθ) complex from excess ε and θ subunits



Separation of the core polymerase (αεθ) complex from excess ε and θ subunits on a 6-ml DEAE-Sephacel (GE Healthcare) column. Successive samples from the peak in the chromatography profile (right) were analyzed by 15% SDS-PAGE (left). Buffers were: buffer R (20 mM Tris.HCl pH 7.6, 0.5 mM EDTA, 2 mM dithiothreitol, 10% v/v glycerol, 100 mM NaCl); buffer B (40 mM Tris.HCl pH 7.6, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% v/v glycerol); buffer S (30 mM Tris.HCl pH 7.6, 0.5 mM EDTA, 2 mM dithiothreitol, 20% v/v glycerol, 100 mM NaCl). To reconstitute the core polymerase, separately purified α[7] and θ[8] were dialyzed in buffer R while ε was left in its storage buffer[8] to avoid unnecessary handling. Proteins were mixed in an α:ε:θ molar ratio of 1:2:3 (total of 16.5 mg of proteins in 30 ml) and left at 4 ˚C for four hours. Excess of ε relative to α and θ relative to ε ensured that all of α in the mixture was in the form of the αεθ complex. To isolate αεθ, the protein mixture was dialyzed into buffer B+50 mM NaCl, and then loaded at 0.5 ml min^{-1} onto a column (1 × 8 cm) of DEAE-Sephacel that had been equilibrated in the same buffer. After the column had been washed with 12.5 ml of buffer B+50 mM NaCl to elute excess of ε and θ, pure αεθ complex was eluted using a linear gradient (40 ml) of 50–650 mM NaCl in buffer B. It eluted in a single peak at ~180 mM NaCl. The purified complex (8 mg) was dialyzed in buffer S and stored at −70 ˚C.

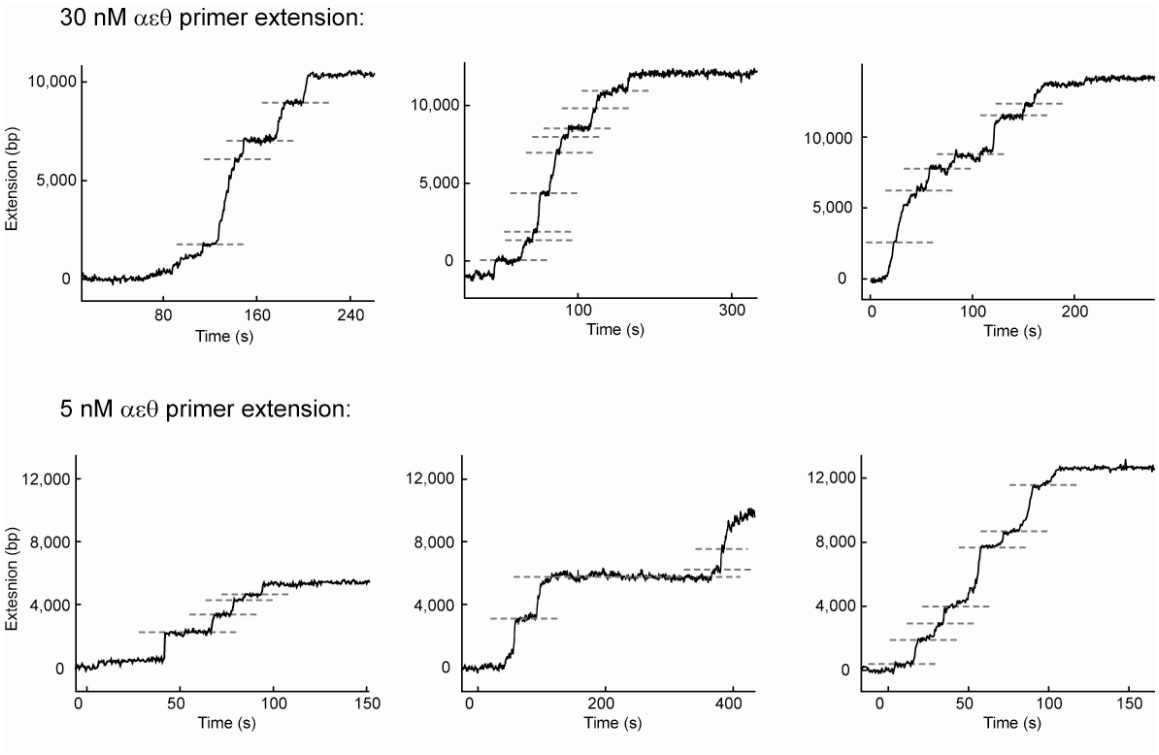
Supplementary Figure 3: Isolation of $\tau_1\gamma_2\delta\delta'\chi\psi$, $\tau_2\gamma_1\delta\delta'\chi\psi$ and $\tau_3\delta\delta'\chi\psi$ clamp loader subassemblies



Isolation of $\tau_1\gamma_2\delta\delta'\chi\psi$, $\tau_2\gamma_1\delta\delta'\chi\psi$ and $\tau_3\delta\delta'\chi\psi$ clamp loader subassemblies on a 1-ml MonoS (GE Healthcare) column. Samples from peaks (indicated by numbers at right) were analyzed by 15% SDS-PAGE (left). Buffers were: buffer R (20 mM Tris.HCl pH 7.6, 0.5 mM EDTA, 2 mM dithiothreitol, 10% v/v glycerol, 100 mM NaCl); buffer A (50 mM Tris.HCl pH 7.6, 5 mM dithiothreitol, 5% v/v glycerol); buffer S (30 mM Tris.HCl pH 7.6, 0.5 mM EDTA, 2 mM dithiothreitol, 20% v/v glycerol, 100 mM NaCl). Constitution and purification of various clamp loaders of DNA Pol III HE was based on a published procedure[6], but with slight modifications that were the consequence of an optimization procedure to maximize the yield of the $\tau_2\gamma_1\delta\delta'\chi\psi$ complex. It was found the use of a τ:γ:δ:δ':χ:ψ molar ratio in the final mixture of 1.8**:**1.0**:**1.0**:**1.0**:**1.2**:**1.2 to be optimal. Purified τ (**Supplementary Fig. 1**), γ[9], δ[7], δ'[7] and $\chi\psi$[10, 11] were separately dialyzed into the reconstitution buffer R. After determination of their concentrations, τ and γ were first mixed to provide a molar ratio of 1.8**:**1.0 and treated at 17˚C for two hours. At the same time, δ was mixed with the $\chi\psi$ complex and δ' in such a way to ensure a final δ:δ':$\chi\psi$ molar ratio of 1.0**:**1.0**:**1.2. The mixture was then treated at 4 ˚C for two hours. Finally, the τ/γ and $\delta/\delta'/\chi\psi$ mixtures were combined (35 mg of total protein in 10 ml) and set aside overnight at 4 ˚C. The equilibrated mixture was clarified by centrifugation (15,000 × *g*; 15 min), split into equal portions and resolved during three separate reproducible chromatography runs using a 1 ml MonoS HR column, as follows. The protein mixture in buffer R was diluted 3-fold with buffer A to reach ~35 mM NaCl in the sample just prior to being loaded at 0.5 ml min^{-1} onto the column that had been equilibrated in buffer A+30 mM NaCl. After the column had been washed with 2 ml of buffer A+30 mM NaCl, the different $\tau/\gamma/\delta/\delta'/\chi\psi$ complexes were eluted using a linear gradient (60 ml) of 30–370 mM NaCl in buffer A. Three complexes of different τ/γ stoichiometry were reproducibly isolated: $\tau_1\gamma_2\delta\delta'\chi\psi$ (eluted at ~115 mM NaCl; 0.60 mg/ml of protein); $\tau_2\gamma_1\delta\delta'\chi\psi$ (at ~150 mM NaCl; 0.70 mg/ml); and $\tau_3\delta\delta'\chi\psi$ (at ~170

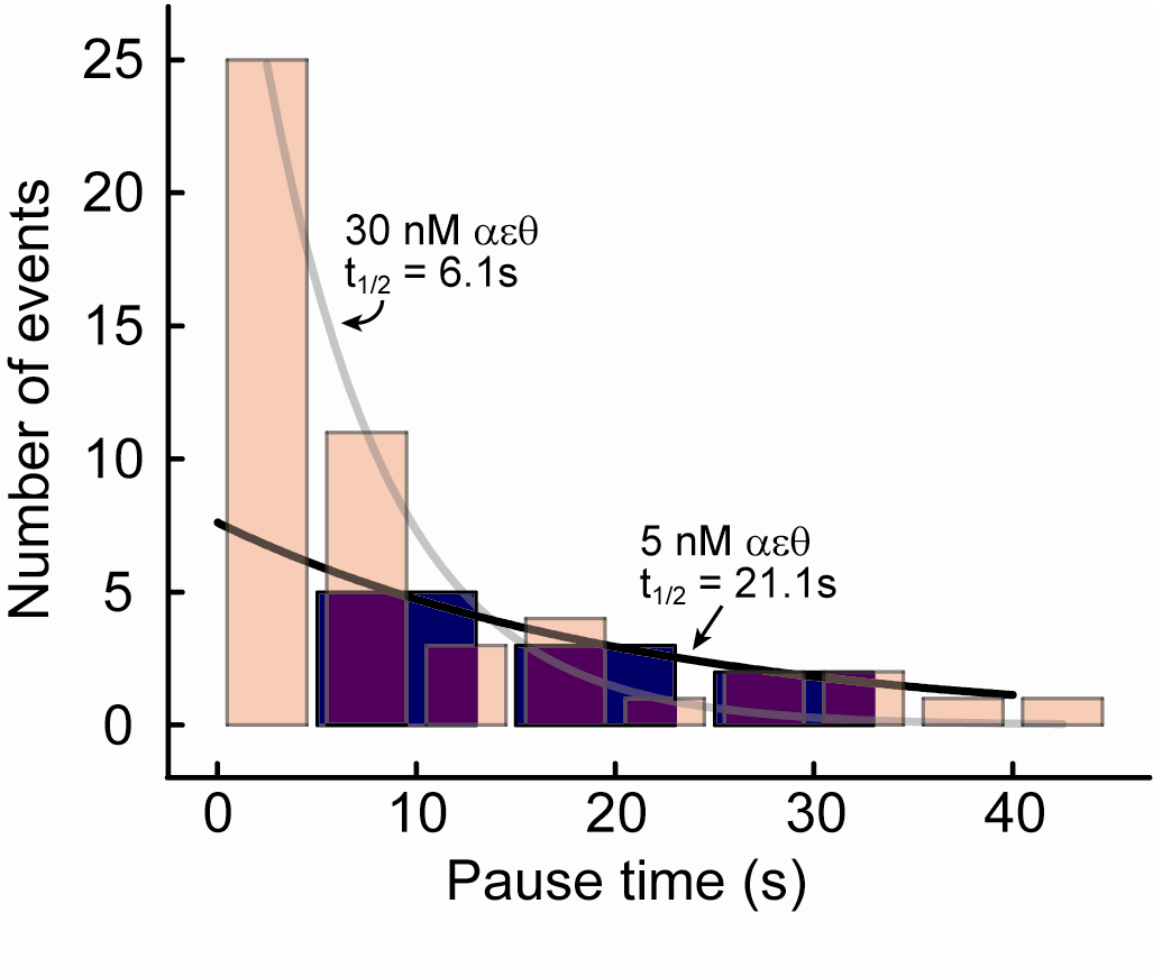
mM NaCl; 0.60 mg ml^{-1}). The isolated complexes were dialyzed in buffer S and stored at −70˚C. Clamp loader subassemblies without the χψ subunits were produced by the same procedure except that χψ was omitted from the constitution step.

Supplementary Figure 4: Primer extension by the Pol III holoenzyme



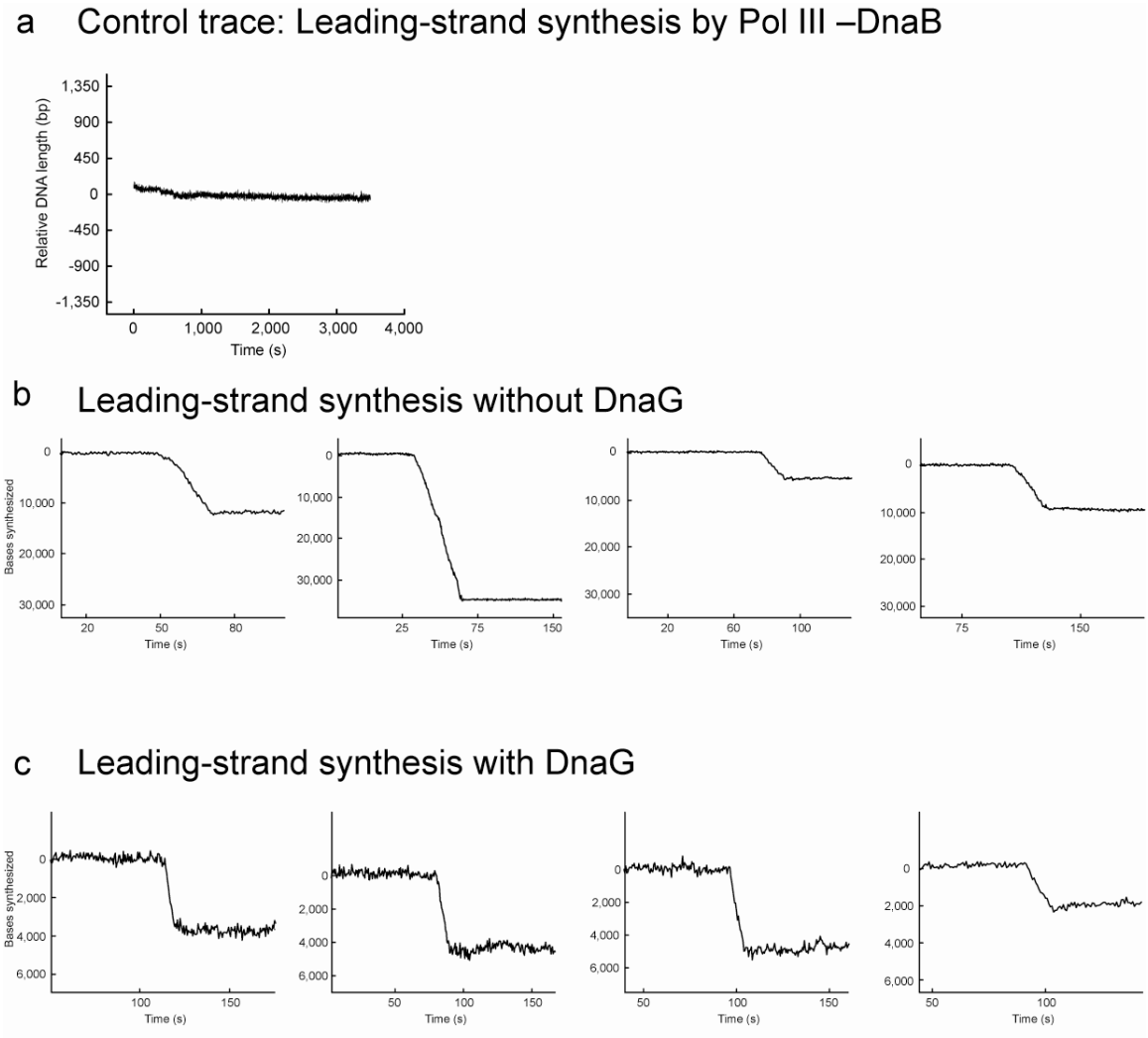
Example traces of primer extension by the Pol III holoenzyme at two different αεθ concentrations. Dashed lines indicate pauses.

Supplementary Figure 5: Primer extension pause time distributions



Distribution of primer extension pause times at 30 nM αεθ (pink with gray fit) and 5 nM αεθ (blue with black fit). Data were fit with a single-exponential decay, and the decay constants were: 6.1 ± 1.5 s at 30 nM αεθ and 21.1 ± 2.6 s at 5 nM αεθ.

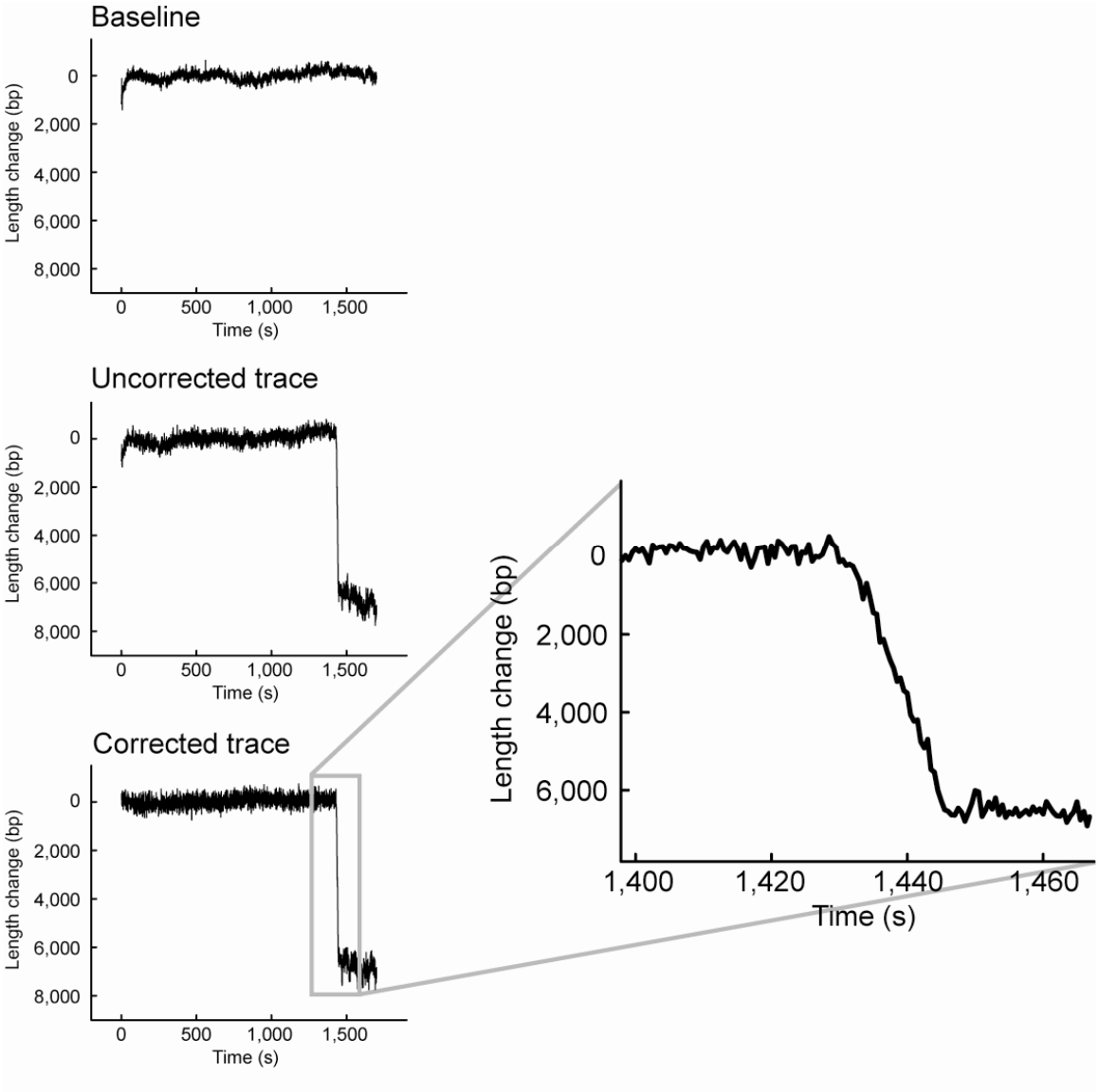
Supplementary Figure 6: Leading-strand synthesis by Pol III and DnaB



a) Leading-strand synthesis by Pol III without DnaB. Since Pol III is not able to synthesize on dsDNA in the absence of DnaB, no length change is observable above the background noise of ~100 bp.
b) Several examples of highly processive leading-strand synthesis by Pol III and DnaB in the absence of DnaG. Traces are shown of various lengths to illustrate the stochasticity of processivity of the complex.

c) Examples of abortive leading-strand synthesis by Pol III and DnaB in the presence of DnaG. Traces illustrate the reduced processivity induced by addition of DnaG to the leading-strand experiments.

Supplementary Figure 7: Data correction



Example of typical data from a single-molecule leading-strand synthesis experiment. DNA length baseline values are determined from a tethered DNA that is not enzymatically altered. This baseline trace is subtracted from altered substrates to remove global flow instabilities from the data. The inset shows a zoomed view of the length change, which appears instantaneous in the overall view (left) due to the compressed time axis, but in fact represents a shortening over many seconds.

Supplementary References:

1. Maki, S. & Kornberg, A. DNA polymerase III holoenzyme of *Escherichia coli*. I. Purification and distinctive functions of subunits τ and γ, the *dnaZX* gene products. *J. Biol. Chem.* **263**, 6547–6554 (1988).
2. Jergic, S., Ozawa, K., Williams, N.K., Su, X.-C., Scott, D.D., Hamdan, S.M., Crowther, J.A., Otting, G. & Dixon, N.E. The unstructured C-terminus of the τ subunit of *Escherichia coli* DNA polymerase III holoenzyme is the site of interaction with the α subunit. *Nucleic Acids Res.* **35**, 2813–2824 (2007).
3. Williams, N.K., Prosselkov, P., Liepinsh, E., Line, I., Sharipo, A., Littler, D.R., Curmi, P.M.G., Otting, G. & Dixon, N.E. *In vivo* protein cyclization promoted by a circularly permuted *Synechocystis* sp. PCC6803 DnaB mini-intein. *J. Biol. Chem.* **277**, 7790–7798 (2002).
4. Dallmann, H.G., Kim, S., Pritchard, A.E., Marians, K.J. & McHenry, C.S. (2000) Characterization of the unique C terminus of the *Escherichia coli* τ *DnaX* protein. Monomeric C-τ binds α and DnaB and can partially replace τ in reconstituted replication forks. *J. Biol. Chem.* **275**, 15512–15519 (2000).
5. Farooqui, A.A. Purification of enzymes by heparin-sepharose affinity chromatography. *J. Chromatogr.* **184**, 335–345 (1980).
6. Pritchard, A.E., Dallmann, H.G., Glover, B.P. & McHenry, C.S. A novel assembly mechanism for the DNA polymerase III holoenzyme DnaX complex: association of δδ' with $DnaX_4$ forms $DnaX_3$δδ'. *EMBO J.* **19**, 6536–6545 (2000).
7. Wijffels, G., Dalrymple, B.P., Prosselkov, P., Kongsuwan, K., Epa, V.C., Lilley, P.E., Jergic, S., Buchardt, J., Brown, S.E., Alewood, P.F., Jennings, P.A. & Dixon, N.E. Inhibition of protein interactions with the β_2 sliding clamp of *Escherichia coli* DNA polymerase III by peptides from β_2-binding proteins. *Biochemistry* **43**, 5661–5671 (2004).
8. Hamdan, S., Bulloch, E.M., Thompson, P.R., Beck, J.L., Yang, J.Y., Crowther, J.A., Lilley, P.E., Carr, P.D., Ollis, D.L., Brown, S.E. & Dixon, N.E. Hydrolysis of the 5'-*p*-nitrophenyl ester of TMP by the proofreading exonuclease (ε) subunit of *Escherichia coli* DNA polymerase III. *Biochemistry* **41**, 5266–5275 (2002).
9. Ozawa, K., Jergic, S., Crowther, J.A., Thompson, P.R., Wijffels, G., Otting, G. & Dixon, N.E. Cell-free protein synthesis in an autoinduction system for NMR studies of protein-protein interactions. *J. Biomol. NMR* **32**, 235–241 (2005).
10. Xiao, H., Crombie, R., Dong, Z., Onrust, R. & O'Donnell, M. DNA polymerase III accessory proteins. III. *holC* and *holD* encoding χ and □. *J. Biol. Chem.* **268**, 11773–11778 (1993).
11. Gulbis, J.M., Kazmirski, S.L., Finkelstein, J., Kelman, Z., O'Donnell, M. & Kuriyan, J. Crystal structure of the chi:psi subassembly of the *Escherichia coli* DNA polymerase clamp-loader complex. *Eur. J. Biochem.* **271**, 439–449 (2004).