**UmuD and RecA* modulate the DNA-binding activity of DNA polymerase IV in *Escherichia coli***

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**Short title:** Regulators of pol IV activity in *E. coli*

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Abstract

Expression of DNA polymerase IV (pol IV) is increased in *Escherichia coli* cells suffering high levels of DNA damage. Despite the resulting increase in intracellular pol IV concentration, the binding of pol IV to the nucleoid does not significantly increase, indicating that pol IV activity is highly regulated in cells. Here, we use quantitative single-molecule imaging to investigate how UmuD and RecA, two binding partners of pol IV regulate its activity in live cells. We find that UmuD acts as a positive regulator of pol IV activity, promoting long-lived association of pol IV with the DNA. In contrast, its cleaved form which accumulates in DNA-damaged cells, UmuD', acts as a negative regulator by inhibiting the binding of pol IV to DNA. In cells treated with the antibiotic ciprofloxacin, up to 40% of pol IV foci colocalise with RecA* nucleoprotein filaments. RecA(E38K), a hyperactive RecA mutant, recruits pol IV to the nucleoid, even in the absence of exogenous DNA damage. *In vitro*, RecA(E38K) forms RecA*-like structures competent of LexA cleavage, even on double-stranded DNA. Using surface plasmon resonance, we show that these RecA*-like filaments can recruit pol IV to double-stranded DNA, consistent with a physical interaction between RecA and pol IV. Together, the results show UmuD and RecA as modulators of DNA binding by pol IV and reveal new elements of a mutagenesis ‘switch’ that occurs in DNA damaged cells. Our findings strongly support the notion that repair synthesis during homologous recombination represents a major function of pol IV in cells.

Author Summary

In *Escherichia coli* cells exposed to DNA damaging agents, DNA repair proceeds through two phases. In the early phase, repair is predominantly error free. If damage persists, cells transition to a mutagenic repair phase. In this study we show that the activity of one repair
enzyme, DNA polymerase IV (pol IV), is modulated during these phases. In the error-free phase pol IV interacts with the UmuD protein, increasing its fidelity during DNA synthesis. We found that UmuD promotes long-lived association of pol IV with DNA, allowing ample time for synthesis to take place. During the mutagenic phase, cells contain the UmuD cleavage product, UmuD'. In addition to forming part of the highly mutagenic pol V enzyme, UmuD' also interacts with pol IV. UmuD' does not increase the fidelity of pol IV, thus DNA synthesis would frequently yield −1 frameshift mutations. UmuD' however reduces this mutagenic potential by inhibiting the binding of pol IV to DNA, limiting its opportunities for DNA synthesis. We further showed that pol IV is recruited to DNA by activated forms of RecA and uncovered fresh evidence in support of double-strand break repair intermediates being major substrates for error-prone pol IV activity.

Introduction

DNA polymerase IV (pol IV), encoded by dinB, is one of three specialised DNA polymerases to be produced at increased levels in Escherichia coli cells suffering DNA damage [1]. In vitro, DNA polymerase IV carries out translesion synthesis (TLS) on a variety of different lesion-containing DNA substrates [2–9]. The most commonly proposed function for pol IV within cells is TLS at stalled replication forks, which may help to maintain chromosomal replication in cells experiencing DNA damage [10,11]. However, in the cell, pol IV spends most of its time at locations distal to replication forks [12]. There is significant evidence that pol IV participates in other pathways, including recombinational repair [13–19] and transcription-coupled TLS [20–23]. However, it remains unclear which of these activities are of most relevance within cells [9].
Single-molecule time-lapse imaging of fluorescently tagged pol IV in live *Escherichia coli* cells revealed that various DNA-damaging agents (ciprofloxacin, UV light, methyl methansulfonate [MMS]) up-regulate the production of pol IV and create binding sites for pol IV [12]. Only 10% of the pol IV binding events (pol IV foci) occurred in the vicinity of replisomes. At late time points during the SOS response (90–100 min after damage induction) pol IV continued to form foci but no longer colocalised with replisomes, even at low levels. This led to the hypothesis that replisome access might be controlled by protein–protein interactions that change around 90–100 min after the induction of SOS. The results also suggest that pol IV function is focused primarily on events that occur away from the replication fork.

The UmuD protein and its cleaved form UmuD′ have a potential role in regulating pol IV activity in cells [24]. The auto-cleavage of UmuD to the shorter form UmuD′ is induced by the cellular recombinase RecA, in particular RecA nucleoprotein filaments (denoted RecA*). UmuD cleavage [25–27] has long been understood to be a key step in the activation of the highly mutagenic enzyme DNA polymerase V (pol V) Mut (UmuD′2C-RecA-ATP; [28]). Several lines of evidence suggest that the conversion of UmuD to UmuD′ might also regulate the activity of pol IV in *E. coli* [20]. Far-Western blots and co-purification experiments indicate that pol IV interacts with UmuD2 and UmuD′2, but not the heterodimer UmuDD′ [20]. Overexpression of pol IV induces high rates of −1 frameshift mutations in cells, which can be suppressed by co-overexpression of UmuD, but not co-overexpression of UmuD′ [20]. Furthermore, UmuD and UmuD′ overexpression reduced frequencies in an adaptive mutagenesis assay compared to an empty vector; overproduction of UmuD even lowered frequencies to equivalent levels of a catalytically dead *dinB* mutant [20]. These observations have led to the proposal that UmuD status regulates the mutagenic activity of pol IV-dependent DNA synthesis. Despite these
advances, it remains unclear if UmuD or UmuD’ affect the fidelity of pol IV or if UmuD and UmuD’ also regulate the binding activity of pol IV, and thus, presumably its processivity.

Beyond this, a series of results indicate that pol IV operates in the repair of double-strand breaks (DSBs) in live cells [15,19,29–33]. Reducing DSB formation (scavenging reactive oxygen species) or introducing defects in the end-resection of double-strand breaks (ΔrecB mutation) greatly reduced the number of pol IV foci that were formed in cells treated with ciprofloxacin or trimethoprim [34]. At end-resected DSBs, RecA nucleoprotein filaments facilitate repair through homologous recombination [35], suggesting that pol IV should colocalise with RecA* structures. A series of observations made by others support this notion.

Pol IV forms a physical interaction with the recombinase RecA in vitro and this interaction modulates the fidelity of pol IV-dependent DNA synthesis [5,24,36]. This interaction is proposed to provide pol IV with the ability to participate in DNA synthesis during RecA-dependent strand exchange reactions [37]. In a fluorescence microscopy study [33], pol IV was shown to colocalise with RecA structures in vivo. However, the RecA-GFP probe that was used to observe RecA localisation does not differentiate between active forms of RecA (i.e. RecA*) and inactive forms, such as storage structures [38]. Furthermore, this RecA-GFP (recA4155-gfp) probe is deficient in recombination, SOS induction and UV survival [39]. It therefore remains unclear whether RecA* structures, such as those that form as intermediates of recombination, represent major or minor substrates for pol IV in cells. With the recent development of a RecA*-specific probe, PAmCherry-mCl [38], we are now in a position to measure pol IV–RecA* colocalisation directly in a time-resolved manner, probing if pol IV predominantly forms foci at homologous recombination hubs.
In this work, we set out to test the following: 1. whether the UmuD cleavage status affects the extent of pol IV focus formation at replisomes and/or the lifetimes of pol IV molecules binding to DNA substrates, and 2. whether pol IV predominantly binds at RecA* structures. Using single-molecule live-cell imaging, here we demonstrated that the DNA binding activity of pol IV is promoted by UmuD in cells treated with the DNA damaging antibiotic ciprofloxacin. In contrast, UmuD’ diminishes pol IV binding. We observed that a large proportion of pol IV foci (up to 40%) colocalise with a RecA* marker. The recA(E38K) mutation (also known as recA730), which constitutively produces RecA*-like activity [40–42], promotes the binding activity of pol IV, even in the absence of DNA damage. We further showed that this activity is due to pol IV interacting with RecA(E38K), which forms RecA*-like structures on single-stranded as well as double-stranded DNA. These findings provide evidence for regulatory roles for both UmuD and RecA in modulating the binding activity of pol IV in E. coli cells. RecA* structures serve as major binding sites for pol IV, demonstrating a major role for pol IV in homologous recombination.

**Results**

**Cleavage of UmuD affects the binding behaviour of pol IV**

Previously, we showed that the limited colocalisation of pol IV foci with replisome markers after treatment with DNA damaging agents drops from ~10% to < 5% (i.e. baseline levels; [12]) at the 90–100 min time-point. Since UmuD₂ and UmuD’₂ physically interact with pol IV and modulate its mutagenic activity [24], we hypothesised that the drop in pol IV–replisome colocalisation that occurs 90–100 min after the onset of DNA damage might be triggered by the cleavage of UmuD to UmuD’.
To investigate the effect of UmuD status on pol IV activity, we compared colocalisation between DinB-YPet and a DnaX-mKate2 replisome marker (coding for a fluorescent fusion of the τ clamp loader protein, serving as a marker for the replisome, τ-mKate2) in four strains (all of which include the dinB-YPet dnaX-mKate2 constructs): i) umuDC+ (EAW643, [12]), ii) ΔumuDC (SSH007), iii) SSH007 expressing the non-cleavable UmuD(K97A) protein from a low-copy plasmid (dinB-YPet dnaX-mKate2 ΔumuDC + pUmuD[K97A], SSH007 + pJM1243), and iv) SSH007 expressing the ‘cleaved’ UmuD’ protein from a low-copy plasmid (dinB-YPet dnaX-mKate2 ΔumuDC + pUmuD’, SSH007 + pRW66). The amount of UmuD(K97A) and UmuD’ produced from each plasmid is 4–5-fold higher than UmuD expressed from its native chromosomal locus [38,43].

Time-lapse movies were recorded for each strain after treatment with ciprofloxacin (30 ng mL⁻¹). At t = 0 min, images of the DinB-YPet signal (300 ms exposure) and τ-mKate2 signal (replisome marker) were recorded for untreated cells. Directly after t = 0, ciprofloxacin was introduced to the flow cell and a time-lapse was recorded over a period of 3 h. The numbers of DinB-YPet foci per cell, reflective of pol IV binding events, were determined at 0, 30, 60, 90 and 120 min time points (Fig 1). Colocalisation between DinB-YPet foci and replisome was also monitored. A complementary set of colocalisation measurements was performed, in which a shorter exposure time of 50 ms was used when collecting the DinB-YPet images in order to better capture transient foci (S1 Fig).

We first monitored pol IV behaviour in cells expressing wildtype levels of UmuD and UmuC (EAW643, Table 1). Cells exhibited no pol IV foci prior to ciprofloxacin treatment (Fig 1A, upper panel), as seen previously [12]. After ciprofloxacin addition, the number of pol IV foci per cell increased to an average of 0.1 foci per cell from 60 min, i.e. one in ten cells exhibited a
pol IV focus. Consistent with our previous observations [12], the percentage of pol IV foci that colocalised with the replisome dropped markedly between the 90 min and 120 min time-points (Fig 1A, middle panel). From 0–90 min after ciprofloxacin addition, 5% of pol IV foci colocalised with replisomes, somewhat less than observed previously. From 120–150 min this decreased to < 2%. The percentage of replisome foci that contained a pol IV focus followed a similar trend (Fig 1A, lower panel); from 0–90 min after ciprofloxacin addition, 0.5% of replisome foci contained a pol IV focus, dropping to ~0.1% (indistinguishable from chance colocalisation) from 120–150 min.

We next examined the effect of deleting the umuDC operon (and thus eliminating UmuD) on the number of pol IV foci and the extent of colocalisation with replisomes (SSH007, Table 1). From 30 min, 10–15% of pol IV foci colocalised with replisomes. Compared to umuDC+ cells, ΔumuDC cells exhibited a 3-fold increase in the number pol IV foci per cell with ~0.3 foci per cell from 60 min after ciprofloxacin addition (Fig 1B, upper panel). Moreover, deletion of umuDC led to a 3-fold increase in the percentage of pol IV foci that colocalise with replisomes (Fig 1B, middle panel). Interestingly, pol IV-replisome colocalisation now persisted above 10% for the 90, 120 and 150 min time points. The percentage of replisomes that contained a pol IV focus was also elevated in the ΔumuDC background (Fig 1B, lower panel). From 30 min, 2–4% of replisomes contained a pol IV focus. Compared to umuDC+ cells, this represents a 6–8-fold increase in colocalisation. These observations suggest that the presence of UmuD (and/or UmuD′) normally suppresses the formation of pol IV foci and also prevents pol IV from binding within, or close to, replisomes.

We next explored the effects of expressing the non-cleavable UmuD(K97A) mutant in dinB-YPet dnaX-mKate2 ΔumuDC cells (SSH007 + pJM1243, Table 1). We note that the
observed effects might be slightly exaggerated because the expression level of UmuD(K97A) from the plasmid is 4–5-fold higher than chromosomal levels [38,43]. At the 90 min time point, cells contained on average 0.6 pol IV foci — a 6-fold increase over umuDC+ cells (Fig 1C, upper panel). This sixfold increase in pol IV foci per cell, was accompanied by a 3-fold increase in colocalisation with replisomes (Fig 1C, middle panel). From 30 min after damage induction, 13% of pol IV foci formed in the vicinity of replisomes. This colocalisation remained relatively constant during the later stages of the SOS response; colocalisation did not drop below 9% from 90–120 min as observed in umuDC+ cells. These observations reveal that UmuD(K97A), and by inference uncleaved UmuD, promote the binding of pol IV to DNA and do not exclude pol IV from replisomes.

During the later stages of the SOS response (90 min after SOS induction), UmuD is converted to UmuD’ [44]. To explore the effects of UmuD’ on pol IV activity, we imaged ΔumuDC cells expressing UmuD’ directly from a plasmid (SSH007 + pRW66, Table 1) at 4-5 times higher expression levels than chromosomal expressed UmuD. These cells produced ~0.1 DinB-YPet foci per cell at 60 min (Fig 1D, upper panel), similar to umuDC+ cells. In the cells expressing UmuD’, colocalisation of pol IV with replisomes was generally low, but highly variable (Fig 1D, middle panel). Two large spikes in colocalisation were apparent at the 30 and 90 min time points. However, due to the low number of foci available for analysis at these time-points, there was very large error associated with these values. No spikes in colocalisation were observed when measuring the proportion of replisomes that contained a pol IV focus (Fig 1D, lower panel). Importantly, the colocalisation of pol IV with replisomes decreased to < 1% after 90 min (Fig 1D, middle panel). Similarly, the percentage of replisomes with that contained a pol IV focus drops between the 90 and 120 min time points (Fig 1D, lower panel). From 30–90 min,
~1% of replisomes contained a pol IV focus. By 120 min < 0.1% of replisomes contained a pol IV focus. Overall, the introduction of UmuD′ into ΔumuDC cells restores rates of focus formation and colocalisation with replisomes to near wild-type (umuDC+) levels.

Taken together, the time-lapse imaging results show that the presence of non-cleavable UmuD results in an increase in pol IV binding events in cells, accompanied by greater access to the replisomes during the late SOS response (90-120 min). Similarly, cells lacking UmuDC exhibit a greater number of pol IV binding events during the late SOS response (90-120 minute window), accompanied by greater access of pol IV to replisomes at these time points. Importantly, the maximum extent of colocalisation of pol IV with replisomes in both cases was similar, with a maximum at ~ 15%. This suggests that even though greater replisome access is permitted in cells lacking UmuDC or carrying the non-cleavable UmuD, replisomes do not represent the major binding substrate for pol IV, during the SOS response. Strikingly, UmuD′ suppresses binding of pol IV to the nucleoid, including at replisomal sites. These results suggest that UmuD cleavage is a biochemical switch for pol IV activity.

**UmuD(K97A) but not UmuD′ provides long-lived replisome access to pol IV**

Time-lapse imaging revealed differences in pol IV activity in umuDC variants with respect to the number of foci per cell and access to replisomes. We noted that in the various DinB-YPet images the foci formed in different strains appeared to exhibit differences in both intensity and shape (Fig 2A–D, first row; 300 ms exposures). For the umuDC+ (Fig 2A), ΔumuDC (Fig 2B) and UmuD′-expressing cells (Fig 2D), most foci were relatively faint and diffuse. In contrast, cells expressing UmuD(K97A) produced brighter, and much more distinct, pol IV foci. Reasoning that these differences might reflect differences in the nature of pol IV interactions with the substrates, we next measured the binding lifetime of pol IV at these sites.
Image sets were recorded during three periods following the addition of ciprofloxacin: 20–45 min, 55–85 min and 120–180 min. For each time interval and each strain (EAW643, SSH007, SSH007 + pJM1243, SSH007 + pRW66; Table 1), burst acquisitions of the DinB-YPet signal were recorded (300 images of 34 ms exposure time, total length of 10.2 s). Subsequently, a corresponding image of the replisome marker τ-mKate2 was collected (see S2B Fig for imaging sequence).

For the umuDC+, ΔumuDC and UmuD'-expressing cells, intensity trajectories collected at replisomal positions predominantly exhibited short-lived binding events (Fig 2, second row). Cells expressing UmuD(K97A), on the other hand, often produced long-lived pol IV binding events. To comprehensively assess the binding lifetimes of pol IV with respect to the UmuD status at sites of replisomes, mean autocorrelation functions were calculated for foci within each strain (Fig 2, third row; S2 Fig). This approach allows us to extract characteristic timescales of signal fluctuations within intensity trajectories, which reflect the lifetimes of binding and dissociation events. Exponential fitting of each mean autocorrelation function gave time constants of $\tau = < 0.03, 0.3$ and 3.3 s, reflecting short-, medium-, and long-lived binding events (S2 Fig). For each strain and time interval after ciprofloxacin addition, the relative proportions of these binding events are plotted in Fig 2 (fourth row).

For both umuDC+ and ΔumuDC cells, most pol IV binding at replisomes is short-lived (Fig 2A, B). In the early stages of ciprofloxacin exposure (25–45 min) the components of the autocorrelation function were 80% short-lived, 10% medium and 10% long-lived. In the later stages, (120–150 min), the proportion of medium-long lived events increased to 40%. In cells expressing UmuD(K97A) long-lived events were much more common: by the 120–150 min period medium and long-lived events comprised 80% of the autocorrelation function (Fig 2C).
stark contrast, cells expressing UmuD′ produced almost exclusively short-lived events (Fig 2D).

UmuD′ appeared to supress the medium and long-lived pol IV binding events that occur in wild-type umuDC+ background following ciprofloxacin treatment.

Taken together, the results indicate that UmuD(K97A) promotes long-lived DNA binding by pol IV, whereas UmuD′ inhibits binding. The deletion of umuDC only marginally increases the binding lifetime of pol IV compared to umuDC+. The results demonstrate that pol IV binding activity is modulated by UmuD and UmuD′ in cells.

Pol IV binds frequently at RecA* structures

Like UmuD and UmuD′, the RecA recombinase modulates the mutagenic activity of pol IV [14,17]. In vitro, DNA synthesis by pol IV is error-prone when operating on D-loop substrates that mimic recombination intermediates [14,17]. Pol IV is known to participate in error-prone DSB repair under a variety of circumstances [13–19,30,32]. It however remains to be determined whether sites of DSB repair represent minor or major substrates for pol IV in vivo.

We determined whether pol IV colocalises with RecA* structures by visualising the localisations of fluorescent pol IV (DinB-YPet) and a RecA* marker (PAmCherry-mCI; a red fluorescent protein fusion of a monomeric C-terminal fragment of the λ repressor that retains the ability to bind RecA* in cells [38]) in SSH092 cells treated with ciprofloxacin — a potent inducer of DSBs [45,46] which also causes the accumulation of reactive oxygen species potentiating killing [47]. Live cell photoactivatable localisation microscopy (PALM) of SSH092 cells treated with ciprofloxacin was performed by collecting images in both channels every 5 min over a period of 3 h following introduction of ciprofloxacin at time point t = 0 min. At each time point, a new field-of-view was recorded.
Following ciprofloxacin treatment, cells typically contained multiple RecA* structures (Fig 3A), including both foci at early time points, and more elongated “bundle” structures described previously at later time points [38]. We next determined the percentage of DinB-YPet foci that colocalised with RecA* features (Fig 3B). Prior to the introduction of ciprofloxacin, RecA* filaments were rarely formed in cells during normal metabolism (< 0.1 mCl foci per cell) consistent with our previous study [38]. Unsurprisingly, we did not detect colocalisation of pol IV with RecA* in untreated cells. Upon introduction of ciprofloxacin to the flow chamber, colocalisation remained low during the early phase of the SOS response (i.e., between 0–45 min after treatment). From 45 min after the introduction of ciprofloxacin, pol IV exhibited extensive colocalisation (10–40%) with RecA* structures in cells. This extensive colocalisation persisted into the late stages of SOS (up to 180 min after treatment). We have previously noted that most of the RecA* foci form at locations distal to the replisome [38].

**RecA* promotes the binding of pol IV to the nucleoid**

Having observed that a large proportion of pol IV colocalises with RecA*, we next set out to determine whether RecA* recruits pol IV to the DNA, characterising the ability of pol IV to bind RecA* structures. Therefore, we utilised a RecA mutant, RecA(E38K), which is able to constitutively induce SOS in cells [40–42,48], suggestive of the formation of RecA* structures being formed in the absence of endogenous DNA damage.

Using surface plasmon resonance (SPR), we here showed that RecA(E38K) forms filaments on ssDNA *in vitro* (S3A, B Fig). Stable association of RecA(E38K) required the presence of ATPγS suggesting that RecA(E38K) forms filaments (S3B Fig). Additionally, RecA(E38K) filaments on ssDNA are competent to cleave LexA (S4 Fig), suggesting that RecA(E38K) forms RecA*-like structures on ssDNA [27,49]. However, in the absence of DNA
damage, we expect exposed ssDNA substrates for RecA(E38K) binding to occur infrequently. Therefore, we additionally tested whether constitutive SOS signalling may occur due to constitutive RecA(E38K)-dsDNA filament formation. To that end, we tested the ability of RecA(E38K) to form filaments on a 60-mer dsDNA substrate. We found that RecA(E38K) binds to dsDNA (S3C, D Fig) and that incubation of dsDNA plasmid substrates with RecA(E38K) promoted LexA cleavage (S4 Fig), indicating that RecA(E38K) forms RecA*-like structures on dsDNA [27,49].

Together, these results allowed us to establish conditions where we could now probe the binding of pol IV to constitutive RecA filaments even in the absence of DNA damage in live cells. We performed single-molecule imaging of dinB-YPet dnaX-mKate2 cells carrying wild-type or mutant alleles of lexA (encoding the SOS-response repressor LexA) and recA (encoding the recombinase RecA). Three strains were examined: i) cells with wild-type lexA and recA alleles (EAW643, dinB-YPet dnaX-mKate2 lexA⁺ recA⁺, Table 1), ii) cells that constitutively express high levels of DinB-YPet even in the absence of exogenous DNA damage (and all other SOS-induced proteins [49]; RW1594, dinB-YPet dnaX-mKate2 lexA[Def] recA⁺, Table 1) and iii) cells that both produce high levels of DinB-YPet and constitutively formed RecA*-like structures (RW1598, dinB-YPet dnaX-mKate2 lexA[Def] recA[E38K], Table 1) [40–42,48].

We set out to determine if the presence of RecA*-like structures formed by RecA(E38K) is sufficient to recruit pol IV to the nucleoid in cells. We recorded burst acquisitions of DinB-YPet motions in the three strains (300 images of 34 ms exposure time, total length of 10.2 s). For each movie, a corresponding image of the replisome marker τ-mKate2 was also captured (see S2A, B Fig for imaging sequence). As expected, with wild-type lexA and recA alleles cells produced few pol IV foci (Fig 4A) [12]. Cells that carried the SOS-constitutive lexA(Def) allele
and the wild-type recA allele produced a relatively high level of DinB-YPet signal, but produced few foci (Fig 4B) [12]. This result is consistent with our previous study in which we concluded that binding is triggered by the presence of damage on the DNA, as opposed to mass action-driven exchange brought on by increased intracellular concentrations of pol IV [12]. In contrast to both recA+ strains, cells carrying both the lexA(Def) allele and the RecA*-constitutive recA(E38K) allele produced both high DinB-YPet signal and readily visible foci (Fig 4C). These results suggest two possibilities: first, that in the absence of ciprofloxacin induced double strand breaks, lexA(Def) recA(E38K) cells exhibit DNA substrates for pol IV that are not present in lexA(Def) cells carrying wild-type RecA; and second, that nucleoid associated RecA(E38K) assemblies are themselves substrates for pol IV in these cells.

We therefore directly tested whether RecA(E38K) interacts with pol IV on filaments assembled dsDNA in vitro. Using an identical SPR experimental setup [38], we assembled RecA(E38K) on a 60-mer dsDNA substrate (S3C, D Fig). We found that pol IV associates with RecA(E38K)-ATPγS filaments formed on dsDNA (S3E Fig) comparing to pol IV association with dsDNA (S3F Fig) Unfortunately, non-specific binding of pol IV to the chip surface, hampered our attempts to extract binding parameters from the sensorgrams. Nevertheless, these results clearly demonstrate that the association of pol IV with the nucleoid is promoted by the presence of RecA*-like structures.

Using the single-molecule data, we next examined fluctuations in the DinB-YPet signals that occur as pol IV binds to, or dissociates from, its binding site. Both replisomal and non-replisomal positions within cells were monitored. Intensity trajectories for DinB-YPet in lexA+ recA+ cells and lexA(Def) recA+ cells predominantly showed short-lived spikes (< 1s; Fig 4D, E), indicative of transient pol IV binding events (milliseconds timescale). In contrast, trajectories for
DinB-YPet in *lexA*(Def) *recA*(E38K) cells often included binding events that were much longer lived (1–10s, Fig 4F), indicative of pol IV binding to its target for longer periods (seconds timescale).

To comprehensively assess pol IV binding lifetimes across all intensity trajectories, mean autocorrelation functions were calculated for each set of trajectories (S2D–F Fig). Fitting of each autocorrelation function give time constants $\tau = < 0.03$, 0.4 and 6.0 s, reflecting short-, medium-, and long-lived binding events (Fig 4G, H; S2G, H Fig). For *lexA*+ *recA*+ cells in the absence of ciprofloxacin, only 1% of replisomes showed evidence of pol IV binding events (Fig 4G, right panel). The normalised mean autocorrelation function for *lexA*+ *recA*+ cells was of low amplitude (0.16 at $\Delta t = 1$ frame, Fig 4G, black line), indicative of there being relatively few long-lived binding events at replisomes across the different trajectories [12]. The *lexA*(Def) *recA*+ background marginally increased pol IV binding activity with 5% of replisomes being visited by DinB-YPet (Fig 4G, right panel) [12]. The autocorrelation function remained of low amplitude (0.3 at $\Delta t = 1$ frame, Fig 4G, black line), indicating that few long-lived pol IV binding events occurred at replisomes in the *lexA*(Def) *recA*+ background. In contrast, *lexA*(Def) *recA*(E38K) cells exhibited a strong increase in pol IV binding activity, both close to and away from replisomes; 31% of replisomes experienced a pol IV binding event (Fig 4G, right panel). The amplitude of the autocorrelation function was also increased (0.4 at $\Delta t = 1$ frame, Fig 4G, black line), indicating that long-lived binding events occurred at replisomes much more frequently. The decay rate of the autocorrelation function had two components (Fig 4G, right panel: $\tau_m = 0.4$ s with an amplitude of 15% and $\tau_l = 6.0$ s with an amplitude of 29%), suggesting that pol IV typically binds in the vicinity of replisomes for periods of a few seconds in the *recA*(E38K)
background. When analysing the binding behaviour of pol IV away from replisomes in these three backgrounds, similar results were obtained (Fig 4H).

**Discussion**

In this study, we arrived at four conclusions: i) UmuD promotes the binding of pol IV to DNA, at both replisomal and non-replisomal sites; ii) UmuD′ inhibits the binding of pol IV to DNA, at both replisomal and non-replisomal sites; iii) pol IV frequently colocalises with RecA* structures; iv) RecA*-like structures promote the binding of pol IV to the DNA. These results lead us to infer that RecA*-like structures can recruit pol IV to the nucleoid. Following ciprofloxacin treatment, this pol IV-RecA* interaction might facilitate pol IV operating at DSB repair intermediates. Further, the RecA* mediated cleavage of UmuD, a biochemical switch that has long been known to regulate pol V activation, also regulates DNA binding by pol IV. The results strongly support roles for both RecA and UmuD as regulatory factors for pol IV in vivo, as proposed previously [5,14,17,24,36].

**UmuD\(_2\) and UmuD′\(_2\) as regulators of pol IV**

A previous study suggested that both UmuD\(_2\) and UmuD′\(_2\) bind to pol IV and modulate its mutagenic activity [24]. Moreover, in vitro experiments have suggested that full-length UmuD binds to the replicative polymerase, α, and destabilises its interaction with the sliding clamp, β, thus facilitating other polymerases, such as pol IV, to access the replisome [50,51].

Here we show that UmuD increases the number of pol IV foci and the binding time of pol IV at the nucleoid. In contrast, UmuD′ inhibits the DNA-binding activity of pol IV. During the first stage of the SOS response, most UmuD is present as full-length UmuD\(_2\). This would promote long-lived binding of pol IV to DNA in a high-fidelity state. Based on rates of pol IV-
dependent DNA synthesis measured *in vitro* (3–5 nt s$^{-1}$; [52]), binding events lasting a few seconds, such as those observed during this study, could permit the incorporation of tens of nucleotides. Based on the high degree of colocalisation we observe between pol IV and RecA*, as well as the strong *recB*-dependence for pol IV focus formation [34], we presume that most of these early events would occur at DSB repair intermediates.

This work allows us to propose the following model for pol IV activity in the context of the SOS response. Cells experiencing extensive DNA damage trigger the full extent of the SOS response, leading to the formation of UmuD′ at late time points after DNA damage. At this point, the cell enters a mutagenic phase. The highly error-prone polymerase pol V Mut becomes active and pol IV, now in the absence of UmuD, introduces –1 frameshift mutations. At the same time pol IV binding becomes infrequent and short-lived in the presence of UmuD′, consistent with an earlier observation that UmuD′ reduces the frequency of adaptive mutagenesis [24]. Thus, while pol IV is error-prone in the presence of UmuD′, mutagenesis would be kept in check by pol IV having reduced access to substrates. This mechanism is in line with the multiple mechanisms that have evolved to restrict the mutagenic activity of pol V [53].

**Pol IV as a recombination protein**

The high degree of colocalisation we observed between pol IV and RecA*, together with the RecA*-like structures promoting pol IV binding events, adds to a growing body of evidence supporting the participation of pol IV in homologous recombination [13–19,29,30,32]. Pol IV colocalises with RecA* (this study) far more frequently than it colocalises with replisomes [12]. In addition, defects in DSB processing strongly suppress both pol IV up-regulation and focus formation [34]. Interestingly, *in vitro*, pol IV is capable of associating with RecA(E38K)-ATPγS filaments formed on dsDNA. These filaments are competent of LexA cleavage, indicative of
RecA*-like structures. *In vivo* in the absence of DNA damage, pol IV forms foci in the
*recA*(E38K) mutant, suggestive of pol IV binding to RecA(E38K) filaments, which presumably
form on dsDNA. An interaction between pol IV and RecA likely facilitates the recruitment of pol
IV to homologous recombination intermediates.

The results presented here indicate that in ciprofloxacin-treated cells the primary pathway
in which pol IV functions in cells is homologous recombination, as opposed to translesion
synthesis at replisomes as is most commonly proposed [11,54,55]. The most likely role for pol
IV in this process is the extension of D-loops during repair synthesis [14,17,36]. The association
of pol IV with RecA is however general [24] and error-prone DNA synthesis by pol IV may not
require RecA* filaments on processed ssDNA intermediates.
Materials and Methods

Strain construction, plasmid construction and transformations

SSH007 is a two-colour strain \((\text{dinB-YPet\ dnaX-mKate2\ \DeltaumuDC})\) derived from EAW643 \((\text{dinB-YPet\ dnaX-mKate2})\). It was made by replacing the wild-type \(\text{umuDC}^+\) gene of EAW643 \(\DeltaumuDC::\text{Cm}\) from RW880 via P1 transduction. Colonies were selected by testing for chloramphenicol resistance.

To investigate the influence of UmuD mutants on pol IV activity, SSH007 was complemented with plasmids that express UmuD(K97A) (pJM1243) or UmuD' (pRW66).

SSH092 was made by transformation; EAW633 \((\text{dinB-YPet})\) carries the pJMuvrA-PAmCherry-mCI vector (see Supplementary Notes for sequence). The PAmCherry-mCI gene block was commercially synthesised and the sequence was verified (IDT gene block). The gene block was introduced into pSC101 [43] using the \text{Apa}\text{I} and \text{Sac}\text{II} restriction sites.

RW1598 was made by P1 transduction of recA730 srlD300::Tn10 from RW244 into RW1594, selecting for TetR. Colonies were then screened for constitutive UmuD cleavage using Western blotting. recA and srlD are about 90% linked.

pJM1243 was made by chemically synthesizing an \text{E.coli} codon optimised umuD(K97A) gene that was cloned into the low-copy spectinomycin resistant vector, pSC101 [43], as HindIII-EcoRI fragment. UmuD(K97A) expression is LexA-regulated. Similarly, pRW66 was made by introducing the umuD' gene into pSC101 [43].

Table 1. Strains used in this study.

| Strain | Relevant Genotype | Parent strain | Source/technique |
|--------|------------------|---------------|------------------|
| MG1655 | \(\text{dinB}^+\ \text{umuDC}^+\ \text{lexA}^+\ \text{recA}^+\) | - | [56] |
|   |   |   |   |
|---|---|---|---|
| RW1594 | *dinB-YPet dnaX-mKate2 sulA::kanR lexA(Def) CmR* | RW1588 | [12] |
| RW244 | *recA(E38K) srlD300::Tn10* | - | [57] |
| RW1594 | *dinB-YPet dnaX-mKate2 sulA::kanR lexA(Def)::CmR recA(E38K) srlD300::Tn10* | Transduction of RW1594 with P1 grown on RW244 |
| EAW633 | *dinB-YPet::kanR* | MG1655 | [12] |
| EAW643 | *dinB-YPet::FRT dnaX-mKate2::kanR lexA+* | EAW633 | [12] |
| RW880 | *ΔumuDC::CmR* | MG1655 | [34] |
| SSH007 | *dinB-YPet::FRT dnaX-mKate2::kanR lexA+ ΔumuDC::CmR* | EAW643 | Transduction of EAW643 with P1 grown on RW880 |
| SSH007 + pJM1243 | *dinB-YPet::FRT dnaX-mKate2::kanR lexA+ ΔumuDC::CmR (chr) + UmuD(K97A) (pl)* | SSH007 | Transformation of SSH007 with pJM1243 |
| SSH007 + pRW66 | *dinB-YPet::FRT dnaX-mKate2::kanR lexA+ ΔumuDC::CmR (chr) + UmuD' (pl)* | SSH007 | Transformation of SSH007 with pRW66 |
| SSH092 | *dinB-YPet::kanR (chr) + PAmCherry-mCI (pl)* | EAW633 | Transformation of EAW633 with pJMuvrA-PAmACherry-mCI [38] |

### Fluorescence microscopy and imaging protocols

For all experiments except for experiments including imaging of PAmCherry-mCI, wide-field fluorescence imaging was performed on an inverted microscope (IX-81, Olympus with a
1.49 NA 100× objective) in an epifluorescence configuration, as described previously [58]. Continuous excitation is provided using semidiode lasers (Sapphire LP, Coherent) of the wavelength 514 nm (150 mW max. output) and 568 nm (200 mW max. output). τ-mKate2 was imaged using yellow excitation light (\(\lambda = 568\) nm) at high intensity (2750 W cm\(^{-2}\)), collecting emitted light between 610–680 nm (ET 645/75m filter, Chroma) on a 512 × 512 pixel\(^2\) EM-CCD camera (C9100-13, Hamamatsu). For DinB-YPet time-lapse imaging, we used green excitation (\(\lambda = 514\) nm) at lower power (240 W cm\(^{-2}\)), collecting light emitted between 525–555 nm (ET540/30m filter, Chroma).

For experiments including imaging of PAmCherry-mCI, imaging was conducted on an inverted microscope (Nikon Eclipse-Ti), equipped with a 1.49 NA 100× objective and a 512 × 512 pixel\(^2\) Photometrics Evolve CCD camera (Photometrics, Arizona, US). NIS-Elements equipped with JOBS module was used to operate the microscope (Nikon, Japan). Continuous excitation is provided using semidiode lasers of the wavelength 405 nm (OBIS, Coherent, 200 mW max. output), 514 nm (Sapphire LP, Coherent, 150 mW max. output) and 568 nm (Sapphire LP, Coherent, 200 mW max. output). DinB-YPet was imaged using green excitation (\(\lambda = 514\) nm) at lower power (~2200 W cm\(^{-2}\)), collecting light emitted between 535–550 nm (ET535/30m filter, Chroma). PAmCherry-mCI was imaged by simultaneous illumination with the activation laser 405 nm (1–5 W cm\(^{-2}\)) and 568 nm readout laser (540 W cm\(^{-2}\)), a PALM (photoactivation localisation microscopy) acquisition protocol, collecting emitted light from 590 nm (ET590LP, Chroma).

Burst acquisitions (movies of 300 × 34 ms frames, continuous excitation with 514 nm light; each frame at 80 W cm\(^{-2}\)) were collected to characterise DinB-YPet binding kinetics; followed by a set of two images (bright-field [34 ms exposure]; mKate2 fluorescence [100 ms exposure].)
Data were recorded from 20–45 min, from 55–85 min and from 120–180 min after ciprofloxacin treatment (30 ng mL\(^{-1}\)). Time-lapse movies were recorded to visualise changes in DinB-YPet binding activity and measure colocalisation with the replisome marker. Sets of three images were recorded (bright-field [34 ms exposure], YPet fluorescence [50 ms exposure]; mKate2 fluorescence [100 ms exposure]) at an interval of 10 min for 3 h. All images were analysed with ImageJ [59].

Time-sampling of DinB-YPet and PAmCherry-mCI expressing cells were performed as follows: First, the bright-field image was taken with 100 ms exposure time. Then, a PALM acquisition protocol (simultaneous illumination with the activation laser 405 [1–5 W cm\(^{-2}\)] and 568 nm readout laser [540 W cm\(^{-2}\)] for 200 frames taken every 100 ms) was used to image PAmCherry-mCI. Third, DinB-YPet was detected using 512 nm laser (50 ms exposure time at \(~2200\) W cm\(^{-2}\)). The experiment was performed over 3 h, time points were sampled at an interval of 5 min. At each time point, a new field-of-view was sampled to minimise laser-induced damage.

To image DinB-YPet and PAmCherry-mCI, sets of three acquisitions were recorded (bright-field [100 ms exposure], YPet fluorescence [50 ms exposure]; PAmCherry fluorescence [simultaneous illumination with the activation laser 405 and 568 nm readout laser for 200 frames each with 100 ms exposure]). This protocol was only executed once for a field-of-view to minimise laser damage. Consequently, each time point shows a new set of cells. The experiment was conducted over 3 h, an image was taken every 5 min.

Flow cell design

All imaging was carried out on cultures growing in home-built flow cells. Imaging was carried out in quartz-based flow cells, similar to those used in our previous study [12]. These
flow cells were assembled from a no. 1.5 coverslip (Marienfeld, reference number 0102222 or 0107222), a quartz top piece (45 × 20 × 1 mm³) and PE-60 tubing (Instech Laboratories, Inc.). Prior to flow cell assembly, coverslips were silanized with aminopropyltriethoxy silane (APTES; Alfa Aesar). First, coverslips were sonicated for 30 min in a 5M KOH solution to clean and activate the surface. The cleaned coverslips were rinsed thoroughly with MilliQ water, then treated with a 5% (v/v) solution of APTES in MilliQ water. The coverslips were subsequently rinsed with ethanol and sonicated in ethanol for 20 s. Afterwards, the coverslips were rinsed with MilliQ water and dried in a jet of N₂. Silanised slides were stored under vacuum prior to use.

To assemble each flow cell, polyethylene tubing (BTPE-60, Instech Laboratories, Inc.) was glued (BONDiT B-482, Reltek LLC) into two holes that were drilled into a quartz piece. After the glue solidified overnight, double-sided adhesive tape was stuck on two opposite sides of the quartz piece to create a channel. Then, the quartz piece was stuck to an APTES-treated coverslip. The edges were sealed with epoxy glue (5 Minute Epoxy, DEVCON home and Epoxy Adhesive, 5 Minute Everyday, PARFIX). Each flow cell was stored in a desiccator under mild vacuum while the glue dried. Typical channel dimensions were 45 × 5 × 0.1 mm.

**Setup of flow cell experiments**

For all imaging experiments, cells were grown at 37 °C in EZ rich defined medium (Teknova) that contained 0.2% (w/v) glucose. EAW643, RW1594 and RW1598 cells were grown in the presence of kanamycin (25 μg mL⁻¹), SH001 cells were grown in the presence of chloramphenicol (25 μg mL⁻¹), SSH007 cells carrying pJM1243 or pRW66 were grown in the presence of spectinomycin (50 μg mL⁻¹). Cells carrying PAmCherry-mC1 were also grown in the presence of spectinomycin (50 μg mL⁻¹). Cells were loaded into flow cells, allowed a few minutes to associate with the APTES surface, then, loosely associated cells were removed by
pulling through fresh medium. The experiment was then initiated by switching the medium to a medium that contains 30 ng mL\(^{-1}\) ciprofloxacin (for cells carrying plasmids: 50 μg mL\(^{-1}\) spectinomycin was added). A flow rate of 50 μL min\(^{-1}\) was applied during the experiment to allow a constant nutrient and oxygen supply by using a syringe pump.

Proteins

The wild-type *E. coli* RecA protein was purified as described [60]. The RecA concentration was determined using the extinction coefficient \(\varepsilon_{280} = 2.23 \times 10^4\) M\(^{-1}\) cm\(^{-1}\) [60].

The *E. coli* RecA(E38K) protein was purified as previously described [61] with the following modifications. After washing the protein pellet with R buffer plus 2.1 M ammonium sulfate, the pellet was resuspended in R buffer plus 1 M ammonium sulfate. The sample was loaded onto a butyl-Sepharose column and washed with 1.5 column volumes of R buffer plus 1 M ammonium sulfate. It was then eluted with a linear gradient from R buffer plus 1 M ammonium sulfate to R buffer, carried out over 5 column volumes. Peak fractions were identified by SDS-PAGE analysis and pooled. The protein was loaded onto a hydroxyapatite column as done previously, but with the linear gradient from 10 to 500 mM P buffer. The fractions were dialyzed against R buffer plus 50 mM KCl and 1 mM DTT three times. The fractions were loaded onto a Source 15S column and washed with R buffer plus 50 mM KCl and 1 mM DTT until the UV trace receded from peak. Next, the pool was loaded onto a Source 15Q column and eluted with a linear gradient from 50 mM KCl to 1 M KCl over 25 column volumes. Peak fractions were identified as above and pooled. A DEAE-Sepharose column was not used. Protein in this pool was precipitated by the addition of equal volume of 90% saturated ammonium sulfate. The precipitate was stirred and then spun down at 13,000 rpm for 30 min. The pellet was resuspended in R buffer plus 1 M ammonium sulfate, stirred for an hour, and then
spun down again. This protein was loaded onto a butyl-Sepharose column and eluted in a
gradient from R buffer and 1 M ammonium sulfate to R buffer. The fractions were identified,
pooled, and concentrated using GE Vivispin 20 10K MWCO centrifuge filter concentrating
units. The protein was flash frozen in liquid nitrogen and stored at -80˚C. The concentration was
determined as above. No exonuclease or other endonuclease activities were detected.

Pol IV (dinB) coding sequence was cloned into NcoI and BamHI sites of pET16b to
generate a native pol IV expression construct. E. coli strain Turner/pLysS (Novagen) carrying
the expression construct was grown in LB medium supplemented with 20 μg/ml
chloramphenicol and 100 μg/ml ampicillin. Expression of pol IV was induced by adding IPTG to
1 mM and growing for 3-4 h at 30˚C. Collected cells (~20 g) were resuspended in 50 mL of lysis
buffer (50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10% sucrose, 2 mM DTT, 1 mM EDTA and
protease inhibitor cocktails). Cells were lysed by lysozyme (2 mg/mL) and the clarified extract
was collected following centrifugation at 15000 x g for 30 min. Pol IV was then precipitated by
ammonium sulfate added to 30% saturation and stirring for 10 min. The precipitate was
subjected to gel-filtration in GF-buffer (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1 mM EDTA, 1
mM DTT) using a GE Healthcare Superdex-75 XK-26/60 gel filtration column. Pol IV fractions
were pooled, dialyzed overnight in PC-buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA 1 mM
DTT, 10% Glycerol), containing 200 mM NaCl and then subjected to phosphocellulose
chromatography (P-11, Whatman). After washing extensively with PC-buffer + 200 mM NaCl,
pol IV was eluted with a linear gradient of 200 mM to 500 mM NaCl. Fractions containing
native pol IV (>99% pure) were pooled and stored at –70˚C.
Surface Plasmon Resonance (SPR) experiments

SPR experiments were conducted on BIAcore T200 instrument (GE Healthcare) using streptavidin (SA) coated sensor chips, probing the formation of RecA structures (assembled from RecA[E38K]) on ssDNA and dsDNA. Experiments were carried out at 20 °C at a flow rate of 5 μL min⁻¹. As described previously [38], SA chips were activated and stabilised, single-stranded biotinylated 71-mer poly-dT oligonucleotide bio-(dT)₇₁ was immobilised, followed by RecA(E38K) filament assembly (**S3A, B Fig**). RecA(E38K) filaments were assembled on bio-(dT)₇₁ by injecting 1 μM RecA(E38K) in SPR^{RecA(E38K)} buffer (20mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM MgCl₂, 0.005% surfactant P20 and 0.5 mM dithiothreitol) supplemented with 1 mM adenosine 5’-(γ-thio) triphosphate (ATPγS) at 10 μL min⁻¹ for 400 s. Similarly, biotinylated dsDNA was immobilised (as previously described [38]), followed by RecA(E38K) filament assembly (**S3C, D Fig**). RecA(E38K) filaments were assembled on dsDNA (sequence: 5’-TCC TTT CGT CTT CAA AGT TCT AGA CTC GAG GAA TTC TAA AGA TCT TTG ACA GCT AGC CAG-3’, 5’ end is biotinylated) by injecting 1 μM RecA(E38K) in SPR^{RecA(E38K)} buffer (20mM Tris-HCl, pH 8.0, 10 mM KCl, 10 mM MgCl₂, 0.005% surfactant P20 and 0.5 mM dithiothreitol) supplemented with 0.5 mM ATPγS at 5 μL min⁻¹ for 500 s. Then, SPR^{RecA(E38K)} supplemented with 0.5 or 1 mM ATPγS buffer was flowed in at 5 μL min⁻¹ for 2,500 s, in order to stabilise the formed filaments. From 3,000 s, 1 μM RecA(E38K) in SPR^{RecA(E38K)} buffer supplemented with 0.5 mM ATPγS was injected at a flow rate of 5 μL min⁻¹ for 4,200 s.

Pol IV association with RecA(E38K)-dsDNA filaments was observed by injecting 0.65 μM pol IV in SPR^{RecA(E38K)} buffer supplemented with 0.5 mM ATPγS for 220 s at 5 μL min⁻¹, monitoring pol IV association (**S3E Fig**). From 220 s, buffer containing 0.5 mM ATPγS was flowed in at 5 μL min⁻¹ and fast dissociation of pol IV was observed. Similarly, pol IV
association with dsDNA was monitored, giving a lower response curve (S3F Fig). We also observed non-specific binding of pol IV to the chip surface, making it impossible to measure binding kinetics of pol IV.

The surface was regenerated as previously reported [38]. Furthermore, the SPR signal were corrected using a flow cell without immobilised bio-(dT)\(_{71}\) or dsDNA and corrected for the amount of immobilised RecA(E38K) [38]. Ghodke et al. utilised this assay to monitor the binding kinetics of mCI at RecA-ssDNA filament [38].

**DNA Substrates for ATPase and LexA Cleavage Assay**

M13mp18 cssDNA was purified as previously described [62], and M13mp18 cdsDNA was prepared as previously described [62–64]. The M13mp18 nicked dsDNA (from here onward called pEAW951) was prepared by nicking with DNaseI according to manufacturer’s recommendations. All DNA concentrations are given in terms of total nucleotides.

**ATPase Assay**

ATP hydrolysis of wild-type RecA and RecA(E38K) on nicked cdsDNA was measured using a spectrophotometric enzyme assay [65,66]. ATP regeneration from phosphoenolpyruvate and ADP was coupled to the oxidation of NADH, which was monitored by the decrease in absorbance of NADH at 380 nm. 380-nm light was used so that the signal remained within the linear range of the spectrophotometer during the experiment. The assays were carried out on a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path length and band pass were 0.5 cm and 2 nm, respectively. The NADH extinction coefficient at 380 nm of 1.21 mM\(^{-1}\) cm\(^{-1}\) was used to calculate the rate of ATP hydrolysis.
The reactions were carried out at 37 °C in a buffer containing 25mM Tris-Ac (80% cation, pH 7.5), 3 mM potassium glutamate, 10 mM magnesium acetate, 5% (w/v) glycerol, 1mM dithiothreitol, an ATP regeneration system (10 units/ml pyruvate kinase, 3.0 mM phosphoenolpyruvate), and a coupling system (2 mM NADH and 10 units/ml lactate dehydrogenase). The concentration of DNA (pEAW951 nicked cdsDNA) was 5µM. One cuvette was a blank control that contained everything except the DNA (volume compensated with TE). The nicked cdsDNA, buffer, and ATP regeneration system were preincubated at 37 °C for 10 min before addition of 3 mM ATP and 3 µM wild-type RecA or RecA(E38K). Data collection was then begun.

LexA Cleavage Assay

The cleavage of LexA was performed essentially as previously described [27]. Reaction mixtures (125µl) contained 40 mM Tris-HCl at pH 8.0, 10 mM MgCl₂, 30 mM NaCl, 2 mM dithiothreitol, 3 µM of M13mp18 circular single-stranded DNA or pEAW951 nicked circular double-stranded DNA, 3 mM ATPγS, LexA, and RecA as noted. Reactions were incubated at 37°C for 10 minutes before addition of LexA. The reaction products were separated and visualized by 15% SDS-PAGE stained with Coomassie blue.

Analysis of colocalisation events of pol IV with replisomes

Foci were classed as colocalised if their centroid positions (determined using our peak fitter tool) fell within 2.18 pixels (218 nm) of each other [67]. For colocalisation analysis, we binned the data in 30 min intervals for a larger sample size per time point due to low numbers of pol IV foci per cell at exposures of 300 ms. We determined that for DinB-YPet–τ-mKate2 localisation the background of pol IV foci expected to colocalise with replisomes purely by
chance is ~4%. This was calculated by taking the area of each cell occupied by replisome foci (including the colocalisation search radius) and dividing by the total area of the cell. The value of 4% corresponds to the mean of measurements made over > 300 cells. As the number of pol IV foci changes in time, the proportion of replisome foci expected to colocalise with pol IV foci by chance also changes in time. At an exposure time of 50 ms, there are almost zero pol IV foci at the beginning of the measurement, thus there is close to zero probability that a replisome focus will colocalise with a pol IV focus by chance. At \( t = 30 \text{ min} \), chance colocalisation is expected to be 5% and at \( t = 120 \text{ min} \), the chance colocalisation is expected to be 3%. At an exposure time of 300 ms, the number of pol IV foci per cell never exceeds ~0.6 foci per cell, thus the level of colocalisation expected to occur by chance is close to zero.

Analysis of pol IV binding kinetics

Replisome localisations were determined by identifying and fitting peaks from \( \tau \)-mKate2 images. From the corresponding burst acquisition movie, the DinB-YPet signal at replisomes was plotted against time (trajectories of DinB-YPet activity at replisomes) from 20–45 min, from 55–85 min and from 120–180 min after ciprofloxacin treatment (S2C Fig). These were divided into trajectories that give and not give pol IV binding events (S2D, E Fig). From this, the percentage of replisomes (\( \tau \)-mKate2 foci) that are visited by DinB-YPet molecules (Fig 4G, right panel) is calculated.

Only trajectories that have pol IV binding events were then used to separate pol IV binding kinetics. The autocorrelation function was applied to each of these trajectories giving signal similarities as a function of the lag time, a method that identifies time-dependent fluctuations in signal dependent on binding and dissociation of molecules. When applying the autocorrelation function to a DinB-YPet trajectory, the correlation of this trajectory with its time
delayed copy is generated for various lag times. With zero lag time, the normalised correlation of
a trajectory with itself is 1. The correlation of a trajectory with its time delayed copy, however,
gives autocorrelation values that range from 0–1 depending on signal fluctuations. DinB-YPet
molecules that are statically bound show no fluctuations in the DinB-YPet fluorescence signal
over time, consistent with the signal being correlated in time. Consequently, the autocorrelation
is between 0–1 for lag times after zero. In contrast, DinB-YPet molecules that are transiently
associated show many fluctuations in the DinB-YPet fluorescence signal over time. The signal is
not correlated in time and results in zero autocorrelation for lag times after zero.

For each time window (20–45 min, 55–85 min and 120–180 min), the mean
autocorrelation function output was calculated to determine the average of DinB-YPet binding
kinetics. The fast decay at t = 0 s corresponds to fast components. From t > 0 s, the curve was
fitted with a two-exponential function where medium or slow components were identified
\( y = y_0 + A_1 \cdot e^{-x/\tau_1} + A_2 \cdot e^{-x/\tau_2} \). Using the \textit{in vitro} experimentally determined rate of nucleotide
incorporation of pol IV as a guide (3–5 nt s\(^{-1}\) [52]), the fast, medium and slow components are
translated to no binding/short-lived binding (unproductive binding), binding events that are
sufficient for the insertion of 1–2 nucleotides or ~10 nucleotides, respectively.

Pol IV binding activity away from replisomes was determined as described above. Pol IV
trajectories were, however sampled, from average projections of pol IV burst acquisitions in time
(average projection over 100 frames, exposure time for each frame was 34 ms; total exposure 3.4
s; see \textbf{Fig 2}, upper row).

\textit{Analysis of colocalisation events of pol IV with mCI}

To measure colocalisation between pol IV and mCI, we first created a maximum
projection of each PAmCherry-mCI movie. Similar to the colocalisation analysis performed for
pol IV with replisomes, foci were classed as colocalised if their centroid positions fell within 218
nm of each other. Chance colocalisation of pol IV with mCI is close to zero at 0 min. Chance
colocalisation is increased from 50 min with ~4%. At 100 min, the chance colocalisation is
~15%.

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Conflict of interest

The authors declare no conflict of interest.
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Table and figure legends

Table 1. Strains used in this study.

Figure 1. Number of DinB foci per cell and colocalisation measurements between DinB and \( \tau \) in different \textit{umuDC} mutants following ciprofloxacin treatment. (A) Upper panel: Number of DinB foci per cell in \textit{umuDC}\(^+\) at 300 ms exposures. Error bar represents standard error of the mean for \( n > 100 \) cells. Middle panel: Colocalisation percentage of DinB with DnaX (green bars) in \textit{umuDC}\(^+\). Time points are binned over 30 min. Error bar represents standard error of the mean between biological triplicates. Lower panel: Colocalisation percentage of DnaX with DinB (magenta bars) in \textit{umuDC}\(^+\). Time points are binned over 30 min. The other columns represent the same measurements as in (A), except from the cell strains (B) \( \Delta \textit{umuDC} \), (C) \( \Delta \textit{umuDC} + \text{UmuD(K97A)} \) expressed from a plasmid, and (D) \( \Delta \textit{umuDC} + \text{UmuD}' \) expressed from a plasmid.

Figure 2. Binding activity of DinB at and away from replisomes in different \textit{umuDC} mutants. (A) Upper panel: Images of DinB and DnaX signal in \textit{umuDC}\(^+\). Left: Projection of DinB signal consistent with 300 ms exposure times. Right: Merged images of discoidal filtered DinB (green) and DnaX signal (magenta). Second panel from the top: Exemplary trajectory showing DinB activity at replisomes in \textit{umuDC}\(^+\). Third panel from the top: Mean autocorrelation function showing DinB activity at replisomes in \textit{umuDC}\(^+\) at 25–45 (light grey line), 55–85 (grey line) and 120–150 min (black line). Error bars represent standard error of the mean over \( > 100 \) trajectories. Bottom panel: Components of the autocorrelation function for DinB at replisomes in \textit{umuDC}\(^+\) showing fast (< 0.03 s, grey), medium (0.3 s, light green) and slow components (1.7 s, dark green). The error bars for slow and medium components were extracted from the fit error using the two-exponential fit (Suppl. Fig 1G, H). The error bar from
the fast components is equivalent to the standard error of the mean from the mean autocorrelation function at lag time 0s. (B) similar to (A), however in $\Delta\text{umuDC}$. (C) similar to (A), however in $\Delta\text{umuDC} + \text{UmuD(K97A)}$ expressed from a plasmid. (D) similar to (A), however in $\Delta\text{umuDC} + \text{UmuD}'$ expressed from a plasmid.

Figure 3. Colocalisation measurement between DinB and mCI after ciprofloxacin treatment. (A) Merged images of discoidal filtered DinB-YPet (green) and PAmCherry-mCI (magenta) at 55, 70, 100 and 155 min after ciprofloxacin addition. (B) Colocalisation percentage of DinB with mCI. Time points are binned over 30 min. Error bar represents standard deviation of biological quadruplicates.

Figure 4. Binding activity of DinB at and away from replisomes in different $\text{lexA}$ and $\text{recA}$ mutants. (A) Images of DinB and DnaX signal in $\text{lexA}^{+} \text{recA}^{+}$. Left: Projection of DinB signal consistent with 300 ms exposure times. Right: Merged images of discoidal filtered DinB (green) and DnaX signal (magenta). (B) similar to (A), however in $\text{lexA}(\text{Def}) \text{recA}^{+}$. (C) similar to (A), however in $\text{lexA}(\text{Def}) \text{recA}(\text{E38K})$. (D) Left: DinB signal at a replisome in $\text{lexA}^{+} \text{recA}^{+}$. Right: DinB signal away from replisome in $\text{lexA}^{+} \text{recA}^{+}$. (E) Left: DinB signal at a replisome in $\text{lexA}(\text{Def}) \text{recA}^{+}$. Right: DinB signal away from replisome in $\text{lexA}(\text{Def}) \text{recA}^{+}$. (F) Left: DinB signal at a replisome in $\text{lexA}(\text{Def}) \text{recA}(\text{E38K})$. Right: DinB signal away from replisome in $\text{lexA}(\text{Def}) \text{recA}(\text{E38K})$. (G) Mean autocorrelation function showing DinB activity at replisomes in $\text{lexA}^{+} \text{recA}^{+}$ (black line), $\text{lexA}(\text{Def}) \text{recA}^{+}$ (grey line) and $\text{lexA}(\text{Def}) \text{recA}(\text{E38K})$ (green line). Error bars represent standard error of the mean over > 100 trajectories. (H) Mean autocorrelation function showing DinB activity away from replisomes in $\text{lexA}^{+} \text{recA}^{+}$ (black line), $\text{lexA}(\text{Def}) \text{recA}^{+}$ (grey line) and $\text{lexA}(\text{Def}) \text{recA}(\text{E38K})$ (green line). Error bars represent standard error of the mean > 100 trajectories.
Supplementary Figure S1. Colocalisation analysis using 50 ms exposures for DinB-YPet and number of DinB-YPet and τ-mKate2 foci per cell. (A) Upper row: colocalisation of DinB with DnaX. Left plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} (green line). Middle plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} + UmuD(K97A) expressed from plasmid (green line). Right plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} + UmuDʹ expressed from plasmid (green line). Bottom row: colocalisation of DnaX with DinB. Left plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} (green line). Middle plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} + UmuD(K97A) expressed from plasmid (green line). Right plot compares \textit{umuDC}$^+$ (black line) with Δ\textit{umuDC} + UmuDʹ expressed from plasmid (green line). Error bars represent standard error of the mean between at least biological triplicates. (B) Number of DinB (upper plot) and DnaX foci per cell (bottom plot) in \textit{umuDC}$^+$ (black line), Δ\textit{umuDC} (red line), Δ\textit{umuDC} + UmuD(K97A) (yellow line) and Δ\textit{umuDC} + UmuDʹ (blue line) after ciprofloxacin treatment. Error bars represent standard error of the mean for \(n > 100\) cells.

Supplementary Figure S2. Burst acquisitions and analysis. (A) Experimental setup. Cells are loaded in a flow cell and immobilised on a positively charged APTES glass surface. Cells were imaged before addition of ciprofloxacin and 25–45, 55–85 and 120–150 min after addition. (B) Burst acquisition sequence. Movies of DinB-YPet were recorded. The movies contain 300 frames at an exposure of 50 ms taken every 100 ms. Subsequently, an image of the τ-mKate2 channel is taken at an exposure time of 100 ms. (C) Exemplary intensity trajectories showing DinB-YPet binding at replisomes. (D) Histogram of DinB-YPet intensities at replisomes. From cut-off to 0: replisomes with no DinB-YPet binding. From cut-off to higher intensities: replisomes with DinB-YPet binding. (E) Grouping of trajectories. Trajectories that show no
DinB-YPet binding at replisomes are excluded from the analysis. Trajectories that show DinB-YPet binding at replisomes are used for the analysis. (F), The mean autocorrelation function is obtained from single autocorrelation function. Each autocorrelation function stems from single intensity trajectories of a DinB-YPet binding event at replisomes. (G), Determining components of autocorrelation functions. The mean autocorrelation function is plotted in black. The autocorrelation function has fast components which consist of noise, short-lived and transient binding events (light grey line). Slower components are fitted with a two-exponential fit (green line) which consist of medium and slow decorrelation events consistent with binding events. (H), Components of the autocorrelation function are plotted in a bar graph. Slow, medium and fast components are indicated by different colours: slow (dark green), medium (light green), fast (light grey). The error bars for slow and medium components were extracted from the fit error using the two-exponential fit. The error bar from the fast components is equivalent to the standard error of the mean from the mean autocorrelation function at lag time 0s.

**Supplementary Figure S3. Sensorgram showing RecA(E38K) filament assembly on ssDNA and dsDNA in order to probe interactions with pol IV.** (A) Sensorgram showing the immobilisation of ssDNA, (dT)$_{71}$, on the SA chip surface (association: dark grey phase; immobilised ssDNA: light grey phase). (B) Following ssDNA immobilisation, buffer containing 1 μM RecA(E38K) (+ 1 mM ATPγS) was flowed into the flow cell, at t = 0 min for 400 s. During this period, RecA(E38K) associated with ssDNA (blue phase), forming a RecA(E38K) filament. At 400 s, buffer containing 1 mM ATPγS was flowed into the flow cell. RecA(E38K) dissociates from the surface (yellow phase). From 1,400 s, RU units are constant, consistent with stabilised RecA(E38K) filaments. (C) Sensorgram showing the immobilisation of dsDNA on the SA chip surface (association: dark grey phase; immobilised dsDNA: light grey phase). (D)
Following dsDNA immobilisation, buffer containing 1 μM RecA(E38K) (+ 0.5 mM ATPγS) was flowed into the flow cell, at t = 0 min for 500 s. During this period, RecA(E38K) associated with ssDNA (blue phase), forming a RecA(E38K) filament. From 500 – 3,000 s, buffer containing 0.5 or 1 mM ATPγS was flowed into the flow cell (yellow phase). From 3,000 – 7,200 s, buffer containing 1 μM RecA(E38K) (+ 0.5 mM ATPγS) was flowed into the flow cell to allow for more RecA(E38K) to associate with the dsDNA. 

(E) Sensorgram showing the association of pol IV with RecA(E38K) structures formed on dsDNA. At t = 0 s, 0.65 uM pol IV (+ 0.5 mM ATPγS) was flowed into the flow cell for 220 s and association of pol IV was observed (green phase). At t = 220 s, buffer containing 0.5 mM ATPγS was flowed into the flow cell (yellow phase). (F) Sensorgram showing the association of pol IV with dsDNA. At t = 0 s, 0.65 uM pol IV (+ 0.5 mM ATPγS) was flowed into the flow cell for 220 s and association of pol IV was observed (green phase). At t = 220 s, buffer containing 0.5 mM ATPγS was flowed into the flow cell (yellow phase). Lower response units are recorded than for the association of pol IV with RecA(E38K) structures on dsDNA.

Supplementary Figure S4. RecA(E38K) forms RecA*-like structures on circular dsDNA.

(A) RecA(E38K) readily binds to dsDNA. In six separate reactions, either RecA(E38K) or wild-type RecA was incubated at 37 °C with nicked circular dsDNA (cdsDNA), ATP, and an ATP regeneration system. (B) LexA Cleavage Assays. Reaction mixtures contained 40 mM Tris-HCl at pH 8.0, 10 mM MgCl₂, 30 mM NaCl, 2 mM dithiothreitol, 3 μM circular single-stranded DNA (cssDNA) or nicked circular double-stranded DNA (cdsDNA), 3 mM ATPγS, LexA, and RecA as noted. Reactions were incubated at 37°C for 10 minutes before addition of UmuD or LexA. The reaction products were separated and visualized by 15% SDS-PAGE stained with Coomassie blue. Lane 1 contains a protein ladder while subsequent groups of three lanes contain
the same reaction mixture sampled at 0, 20, and 40 minutes. On cssDNA, RecA(E38K) and wild-type RecA form RecA* structures. On cdsDNA however, RecA(E38K) forms RecA*-like structures in contrast to wildtype RecA.

Supplementary Notes

Sequence of pJMuvrA-PAmCherry-mCl vector:

AAGCTGGAAGATCTTCCCTGGCACACGACAGGTCTTTCCCGGACTGGAAGACGCGGAGTGA
GCGCAACGCAATTAATGTGAGTGTAGCTCACTCATATTAGCACCCACCCGAGGCTTTACACTT
TATGCTTCCGGGCTCGTATGTTGTGGAATTGTGAGCGGATAACAAATTTTCACACAGG
AACACGCTATGACCATTACGCGCAAACTACTCCTCAACTAACCTCAAACCTG
AAAAAGCTGGGTACCGGGCCCCCCCTTCGAGTGAGCTTTCCCGGGAACAAACCTGGCC
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GCCACGTGTTTCGAGATCGGCCCGGGCGAGGAGGGCGAGGGGAGCCCCCTACGAGG
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CGAGGACGGCCGCGTGGTGACCCGAGGACTCTCCTCCCTGCAAGACGGTGAGT
TCATCTACAAGCTGCGCGGCACCAACTTCCCCTCCGCAGCCGACAGG
AGAAGAAGACCCTGGGGTCGAGGGCCCTCTCCGAGCGATGATTCCCGAGGAGGC
GCCCTGAAGGGCAGGGTGCAAGCTGAGCAGGGCGCAGGGCGCCACTACG
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CCTGACGGAATGTTAAATTTCTCCTGTTGACCCCTGAGCGCGGCTGTAGGCGGATTTTC
TGCAATTGCCGCCCTGTGGGTTGAGATGTTTACCTTCGCAGAAAATCGATCCGCGATAGC
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TTCTATAGTTTCTAGAGAATAGGAAATTCGATCTTTTGAATTGAAAACCTCATCGAGATC
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TCGGTCAAAATAAGGTATTACAGTAGGAATATACCATGAGTGACGTGAATCCGAG
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GAGGCCCGACCGATCGCCCTTCACAGGCCTTGAGCTGGAGAATGGGA
CGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGGTGTTTGTTAGTTACGCAGCGGTA
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AATAAGCCCTACAAATTGGGAGATATATCATGAAAGGCTGTTTCTTGTTAT
CGCAGATGTTGCGAGAATCGCAACATCCGCAATTAAATCTAGCGAGGCTTTAC
T
Figure 1

A. umuDC^+  
dinB-YPet dnaX-mKate2 umuDC^+

B. ΔumuDC  
dinB-YPet dnaX-mKate2 ΔumuDC

C. umuD(K97A)  
dinB-YPet dnaX-mKate2 ΔumuDC
UmuD(K97A) plasmid (4-5 x higher expression)

D. umuD'  
dinB-YPet dnaX-mKate2 ΔumuDC
UmuD' plasmid (4-5 x higher expression)

DinB (pol IV) foci
DinB-YPet foci per cell with 300 ms exposures

Colocalisation (%)
Percentage of DinB-YPet foci that colocalise with a τ-mKate2 focus with 300 ms exposures

Colocalisation (%)
Percentage of τ-mKate2 foci that colocalise with a DinB-YPet focus with 300 ms exposures

Time (min) after ciprofloxacin addition
Figure 3

A. Merged images: DinB-YPet and PAmCherry-mCl

- Ciprofloxacin at 30 ng/mL
  - 55 min
  - 70 min
  - 100 min
  - 155 min

B. Colocalisation: DinB (pol IV) foci that contain an mCl focus

Colocalisation (%)

Time (min)
Figure 4

A. lexA<sup>+</sup> recA<sup>+</sup> (dinB-YPet dnaX-mKate2 lexA<sup>+</sup> recA<sup>+</sup>)

B. lexA<sup>(Def)</sup> recA<sup>+</sup> (dinB-YPet dnaX-mKate2 lexA<sup>(Def)</sup> recA<sup>+</sup>)

C. lexA<sup>(Def)</sup> recA(E38K) (dinB-YPet dnaX-mKate2 lexA<sup>(Def)</sup> recA(E38K))

Images:
DinB (pol IV) signal and replisome signal

Images:
D. lexA<sup>+</sup> recA<sup>+</sup> (DinB signal at a replisome)
E. lexA<sup>(Def)</sup> recA<sup>+</sup> (DinB signal at a replisome)
F. lexA<sup>(Def)</sup> recA(E38K) (DinB signal at a replisome)

Exemplary trajectories:
DinB (pol IV) signal at and away from replisomes

Exemplary trajectories:
D. lexA<sup>+</sup> recA<sup>+</sup> (DinB signal at a replisome)
E. lexA<sup>(Def)</sup> recA<sup>+</sup> (DinB signal away from a replisome)
F. lexA<sup>(Def)</sup> recA(E38K) (DinB signal away from a replisome)

Mean autocorrelation function:
DinB (pol IV) signal at and away from replisomes

Mean autocorrelation function:
D. lexA<sup>+</sup> recA<sup>+</sup> (Auto correlation)
E. lexA<sup>(Def)</sup> recA<sup>+</sup> (Components %)
F. lexA<sup>(Def)</sup> recA(E38K) (Auto correlation)
G. lexA<sup>(Def)</sup> recA(E38K) (Components %)

Exemplary trajectories:
DinB signal at and away from replisomes

Exemplary trajectories:
D. lexA<sup>+</sup> recA<sup>+</sup> (Auto correlation)
E. lexA<sup>(Def)</sup> recA<sup>+</sup> (Components %)
F. lexA<sup>(Def)</sup> recA(E38K) (Auto correlation)
G. lexA<sup>(Def)</sup> recA(E38K) (Components %)