The RecBC complex protects the nascent DNA in the presence of single-stranded DNA gaps

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ABSTRACT

Following the encounter with an unrepaired DNA lesion, replication is halted and can restart downstream of the lesion leading to the formation of a single-stranded DNA (ssDNA) gap. To complete replication, this ssDNA gap is filled in by one of the two known lesion tolerance pathways: the error-prone Translesion Synthesis (TLS) or the error-free Homology Directed Gap Repair (HDGR). In the present work, we show a role for the RecBC complex that is distinct from its canonical function in homologous recombination at double strand breaks: RecBC (independently of the RecD subunit) is required to protect the 3'-end of the nascent DNA at the lesion site, in order to promote efficient lesion bypass. In the absence of RecBC, the nuclease ExoI can access and degrade the nascent DNA, thus strongly inhibiting the action of the TLS DNA polymerases and also affecting the HDGR mechanism. RecBC is recruited only when post-replicatively ssDNA gaps are formed and are covered by the single-stranded binding protein (SSB). This protective role of RecBC is reminiscent of BRCA2's role in human cells, once again highlighting the evolutionary conservation of DNA replication mechanisms across all living organisms.

INTRODUCTION

DNA replication is constantly challenged by different types of roadblocks that can transiently slow down or stall replication. Unrepaired lesions are one of these major roadblocks all organisms have to face. Several works in *Escherichia coli* have demonstrated that in the presence of unrepaired lesions the progression of the replicative helicase DnaB is not halted (1–3) and replication can restart ahead of the lesion (1, 4, 5), resulting in lesion skipping and the formation of a single-stranded DNA (ssDNA) gap (6–8). In order to complete replication, the ssDNA gap is filled in by one of the two known lesion tolerance pathways, identified both in prokaryotes and eukaryotes: 1) Translesion Synthesis (TLS), which employs specialized low-fidelity DNA polymerases that insert a nucleotide directly opposite to the lesion, with the potential to introduce mutations; 2) Homology Directed Gap Repair (HDGR), which uses the information of the sister chromatid to bypass the lesion in a non-mutagenic way through homologous recombination mechanisms (9–11). Regulation of these two pathways is fundamental to define the level of mutagenesis during lesion bypass. Our laboratory has previously shown that several factors contribute to regulate the balance between HDGR and TLS: the nature of the DNA lesion (12, 13), the amount of TLS polymerases present in the cell (14) and the density of lesions (15). Very recently, we have also shown that the processing of the ssDNA gap during the presynaptic phase plays a role in the regulation of lesion tolerance pathways (16).

Interestingly, we have observed that loss of *recB* gene has an impact on lesion tolerance pathway, in particular it leads to a strong decrease in TLS events, and this independently of the nuclease and the RecA loading activities of RecB (13, 17). RecB is part of the RecBCD complex, also known as exonuclease V, a heterotrimeric complex that plays a role in several aspects of cell metabolism and genome maintenance, such as double-strand break repair, conjugation, chromosomal segregation, degradation of foreign DNA and completion of DNA replication (see (18–20). In particular, RecBCD is the key factor in homologous recombination pathway, also referred to as the RecBCD pathway (21–23). The complex is composed of three subunits: RecB is the main subunit, containing a DNA-dependent ATPase activity, a 3’->5’ DNA helicase activity, the nuclease active site and the RecA interaction domain (24). RecC itself does not possess any catalytic activities, but it does bind ssDNA, and

it can stimulate the ATPase and helicase activities of the RecB protein, with which it forms a complex of 1:1 stoichiometry (25). The main role of RecC is to recognize a specific sequence, the χ (Chi) site (26–28), that participate in the regulation of the complex. RecD is the smallest subunit and possesses a ssDNA-dependent ATPase activity and 5'->3' DNA helicase activity (29) (30). The other functions of RecD are to regulate the nuclease activity of RecB, to increase the processivity of the complex and to negatively regulate RecA loading (31–34). The preferred substrate of the RecBCD complex is a blunt or nearly blunt double strand DNA ends: the RecB subunit binds to the 3' end while the RecD subunit associates with the 5' extremity (35–37). Individually, each subunit is a poor ATPase, helicase or nuclease, but together they form one of the most processive helicase/nuclease machine (37–39).

Initially, the complex was thought to contain only RecB and RecC subunits (40), later RecD was identified as the subunit regulating the nuclease activity and containing a helicase activity (41–43). Whereas *recD* null mutants display normal cell viability, resistance to DNA damaging agents and are recombinant proficient, mutations in *recB* or *recC* genes strongly affect cell viability, reduce conjugal recombination 100- to 1000-fold, and sensitize cells to DNA-damaging agents (18). This is due to the fact that in the *recD* strain, the nuclease activity can be substituted by the nuclease RecJ (43) and that RecA is constitutively loaded, independently of the χ site (32). Since the single *recB* and *recC* mutant strains resulted in identical phenotypes, this suggested that the minimal complex was RecBC. However, *in vitro* RecBC and RecBCD complex do not possess the same enzymatic properties: several works showed that compared to the potent and processive nuclease/helicase RecBCD, RecBC has no exonucleolytic activity on a linear substrate and its endonucleolytic activity is strongly inhibited, as well as its helicase activity. Instead, the DNA-dependent ATPase activity of RecBC is preserved, in particular in the presence of a ssDNA substrate (24, 29, 44). Therefore, it is possible that the two complexes exist in the cell with different physiological functions, however, no specific cellular role has been associated to the sole RecBC complex up to now.

In this work, we show that the RecBC complex is required to protect the nascent DNA when a ssDNA gap is formed after lesion skipping and repriming. The RecBC complex will prevent the nuclease attack of ExoI, most likely by binding to the ds-ssDNA junction of the ssDNA gap. In the absence of RecBC, Exo I action on the nascent DNA turns out to strongly inhibit TLS and also affect HGDR mechanism. Therefore, RecBC complex is necessary to allow

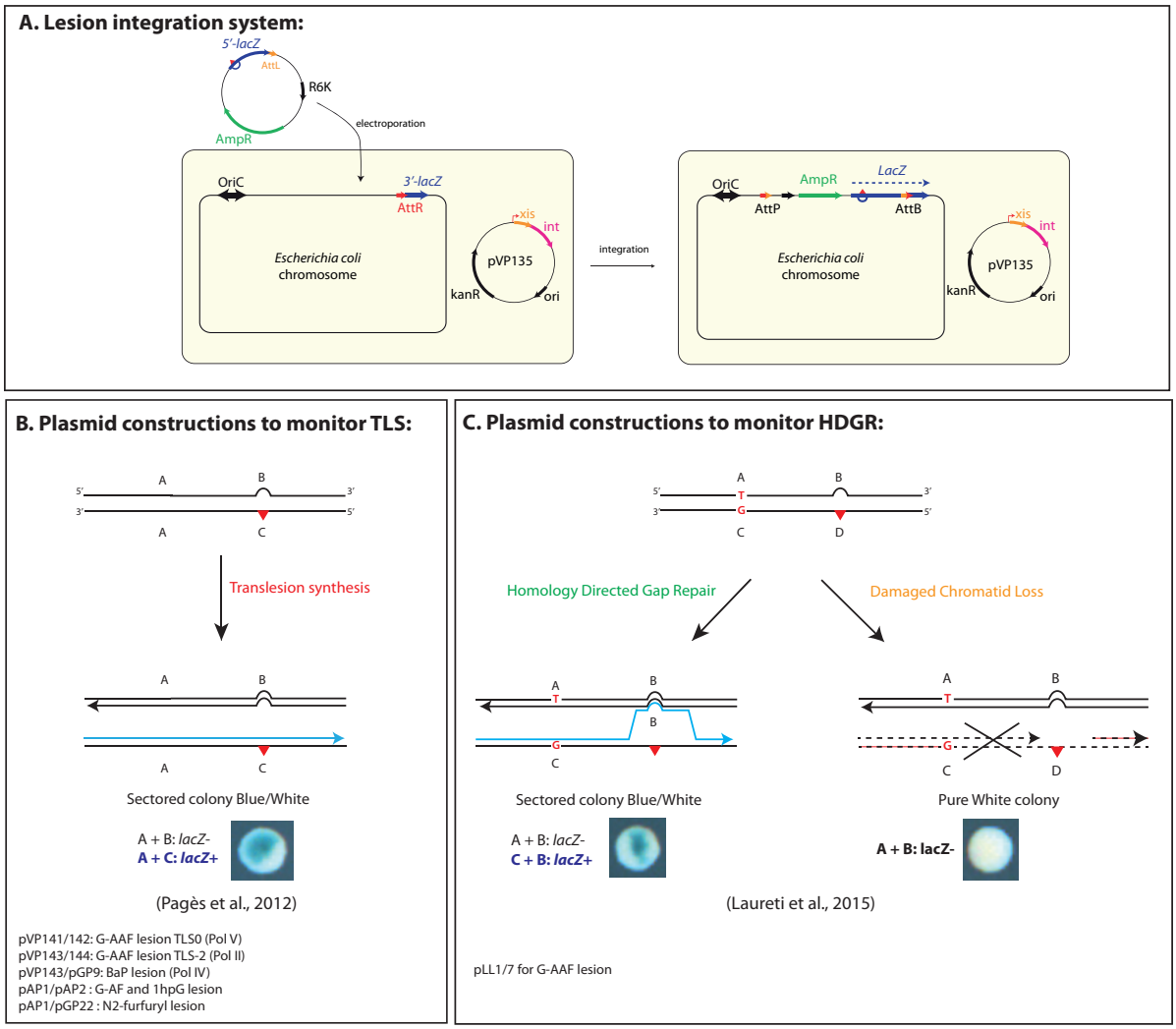
efficient lesion bypass, a new role independent of its canonical role in double strand break repair.

RESULTS

We have previously shown in the presence of a UV photoproduct lesion (*i.e.* TT6-4) that deletion of *recB* gene affects lesion tolerance pathways, decreasing HDGR mechanism and strongly inhibiting TLS polymerases (13, 17). We have also shown that this role in lesion bypass is independent of the nuclease and the RecA loading activity of RecB and more interestingly, that the RecB participation to lesion bypass does not involve processing of a double-strand break that could potentially arise from the presence of a lesion (17). In this study, we further precise this unexpected role of RecB.

To monitor lesion tolerance mechanisms, we took advantage of our previously described genetic assay (see Suppl Fig 1A) that allows to insert a single replication-blocking lesion into the chromosome of *E. coli,* either on the lagging or in the leading orientation regarding the replication fork direction. TLS or HDGR events are visualized and measured by a colorimetric assay based on the *lacZ* reporter gene using specific lesion integration plasmids (Suppl Fig 1B and 1C) (12, 13, 45). This assay allowed us also to reveal another lesion tolerance strategy, that we named Damaged Chromatid Loss (DCL) that promotes cell proliferation by replicating only the undamaged chromatid at the expense of losing the damaged chromatid (13) (see Suppl Fig 1C). In the present study, we mainly used the chemical guanine adduct N-2-acetylaminofluorene (G-AAF) positioned in the *Nar*I hotspot sequence: in this configuration the lesion can be bypassed either by the TLS polymerase Pol V in an error-free manner (indicated as TLS0 events), or by the TLS polymerase Pol II, inducing a -2 frameshift (indicated as TLS-2 events) (46).

The *E. coli* strains used are deficient for the mismatch repair system (*mutS*) to prevent correction of the genetic markers of the integrated plasmids, as well as for nucleotide excision repair (*uvrA*), to avoid excision of the lesion and to focus on lesion tolerance events.



Suppl Fig 1. Lesion tolerance assay and lesion containing plasmids.

A) The system is based on the phage lambda site-specific recombination and it requires a recipient *E. coli* strain with a single *att*R site fused to the 3'-end of the *lacZ* gene and a non-replicating plasmid construct containing a single DNA lesion (red triangle) in the 5'-end of the *lacZ* gene fused to the *att*L site. The recombination reaction between *att*L and *att*R is controlled by ectopic expression of phage lambda *int–xis* proteins (provided by pVP135), and leads to the integration of the lesion-containing vector into the chromosome. Integrants are selected on the basis of their resistance to ampicillin and the chromosomal integration restores an entire *lacZ* gene.

B) Plasmid duplexes used to monitor TLS events: the non-damaged strand (A+B markers) contains a short sequence of heterology opposite the lesion that inactivates the *lacZ* gene and serves as a genetic marker for strand discrimination. Only the replication by TLS polymerases of the A+C markers will give rise to a functional *lacZ* gene and therefore to a sectored blue-white colony.

C) Plasmid duplexes used to specifically monitor strand exchange mechanisms, *i.e.* HDGR events: four genetic markers have been designed in order to distinguish the replication of the non-damaged strand (containing markers A and B) from the damaged strand (containing markers C and D). Using a combination of frameshift and stop codon, we inactivated *lacZ* gene on both the damaged (C-D) and undamaged (A-B) strands of the vector. Only a strand exchange mechanism by which replication has been initiated on the damaged strand (incorporation of marker C), and where a template switch occurred at the lesion site (leading to incorporation of marker B) will restore a functional *lacZ* gene

leading to sectored blue-white colonies. When the damaged strand is lost, only the A and B marker will be replicating giving rise to a white colony.

RecBC complex but not RecD participates in lesion bypass

Since RecB is part of the RecBCD complex, we decided to test whether all the subunits of the complex were involved in lesion bypass. We inactivated separately *recB, recC* and *recD* genes and measured tolerance events (*i.e.* HDGR, TLS and DCL) in the presence of a G-AAF lesion. Deficiency in *recB* gene resulted again in a decrease of HDGR, loss of Pol II TLS events (down to <0.4%) and an increase of DCL events (Fig 1), confirming the previous results obtained with the UV lesion (13, 17). A *recC* deficient strain behaved like the *recB* strain (Fig 1). We observed the same phenotype for lesion bypass whether we inactivated *recB* or *recC*, similarly to *recB* or *recC* inactivation regarding double strand break processing. On the other hand, no decrease in HDGR or TLS was measured for the *recD* deficient strain (Fig 1), suggesting that only the RecBC complex is required for lesion bypass. RecD subunit is involved in the regulation of the nuclease activity of RecB (31, 33). Since we already showed that the nuclease dead allele of RecB ($RecB^{D1080A}$) has no effect on HDGR or TLS (17), it is not surprising that RecD subunit is not required.

Further confirmation that RecBC, without RecD, is involved in lesion bypass came using a plasmid construction containing two lesions on the opposite strands: this configuration induces a two-fold increase in TLS events due to the generation of overlapping ssDNA gaps that prevent HDGR (15). Deletion of the *recB* gene had a strong effect on TLS levels, while deletion of *recD* gene or the nuclease dead allele of *recB* behaved as the parental strain (See Suppl Figure 2).

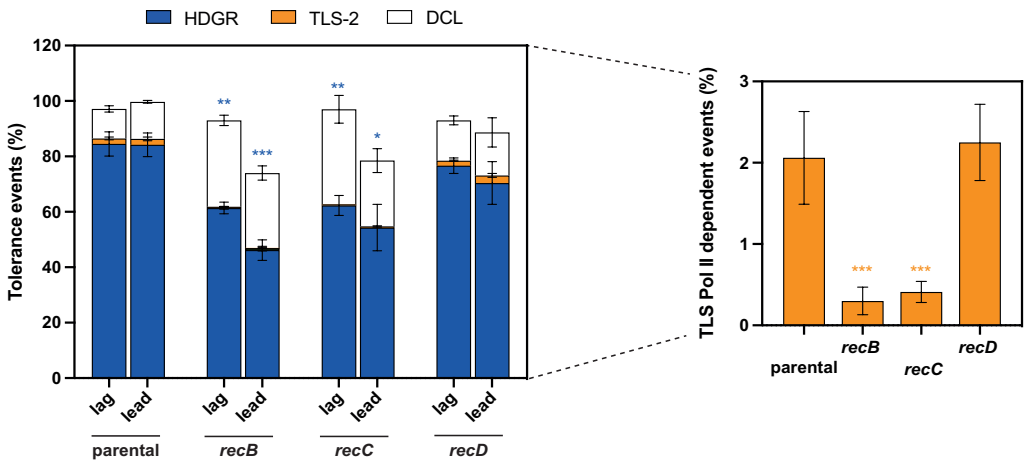
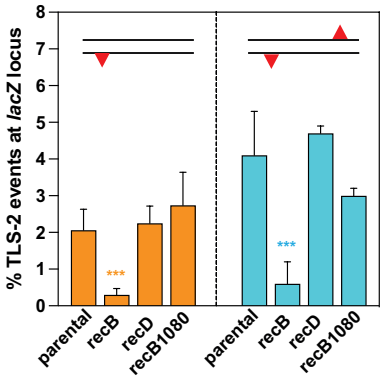


Figure 1. The RecBC complex, without RecD, is involved in lesion bypass.

The graph represents the partition of lesion tolerance pathways, that are Homology Directed Gap Repair (HDGR), Translesion Synthesis (TLS) and Damaged Chromatid Loss (DCL), as well as cell survival in the presence of a G-AAF lesion in the mutants of the three subunits of the RecBCD complex (for more details see Material & Methods section). The lesion was inserted either in the lagging (lag) or in the leading (lead) strand. For TLS-2 events, the data for lagging and leading have been pooled together since no difference has been observed. The blue and orange asterisks represent the statistical significance for HDGR and TLS-2 events, respectively, for every mutant strain compared to the parental strain.

The data in every graph represent the average and standard deviation of at least three independent experiments. Statistical analyses have been performed with Prism using an unpaired *t*-test. * $p < 0,05$; ** $p < 0,005$; *** $p < 0,0005$.

NB: The TLS data for the parental and *recB* strains have been already published in (17).



Suppl Figure 2. The graph represents the Pol II TLS events at a single G-AAF lesion located in the *lacZ* gene in the absence (orange colour) or in the presence of an additional lesion (light blue colour) on the opposite strand. The data in every graph represent the average and standard deviation of at least three independent experiments.

Exonuclease screen in the *recB* deficient strain

In the absence of RecBC, HDGR mechanism is decreased and TLS is severely impaired. From these data, we hypothesized that RecBC could be involved in the protection of the nascent DNA blocked at the lesion site from nuclease degradation, similar to what has been described in human cells where BRCA2, together with RAD51, protects against the MRE11 nuclease attack (47, 48). Degradation of the 3'-end of the nascent DNA is detrimental for TLS polymerases since their cognate substrate is missing and no recruitment will be possible. In addition, important degradation of the nascent DNA will also impact the efficiency of HDGR mechanism. To corroborate our hypothesis, we performed a genetic screen to identify the putative nuclease(s) responsible for this degradation in the absence of *recB*. We looked for

candidate genes that encode exonucleases with a 3'->5' orientation that preferentially degrade single-stranded DNA and that have a role in DNA metabolism and genome maintenance. Based on the literature (see reviews (49, 50)), we chose to test ExoI (also known as XonA/SbcB), ExoVII, ExoX and SbcCD.

We inactivated singularly each exonuclease in the *recB* deficient strain and we measured Pol II dependent TLS events in the presence of a G-AAF lesion in order to see whether we could restore the level of the parental strain. From this first screen, ExoI and SbcCD emerged as good candidates, since deletion of *exoI* and *sbcCD* in the *recB* strain recovers fully or partially, respectively, the level of TLS-2 (Fig 2A).

ExoI prevents efficient lesion bypass in the absence of RecBC

Previously, we have shown that the deletion of *recB* affects lesion bypass of all three TLS polymerases (17). As we have mentioned, when positioned in the *Nar*I hotspot sequence, the G-AAF lesion can be bypassed by Pol II, but also by Pol V. However, under physiological conditions, Pol V bypass is very low (<0.5%) (12). Hence, to assess the role of ExoI and SbcCD on Pol V TLS events, we used a plasmid expressing both the active form of Pol V (UmuD'C in which UmuD is cleaved (51, 52)) and Pol II under their own promoters (14). The expression level of both TLS polymerases is 3-5 times higher than in the parental strain (due to plasmid copy number), which results in an increase of Pol II TLS events and in a stronger increase of Pol V TLS events, from 0.5% to more than 20% (Fig 2B). These conditions allow a better read-out to monitor the effect of the nucleases on both Pol V and Pol II mediated TLS. As expected, deletion of the *recB* gene strongly decreased the level of both Pol II and Pol V dependent TLS events (Fig 2B). While deletion of the nuclease *sbcCD* in the *recB* strain did not restore TLS levels, the double mutant *exoI recB* fully or partially recovered respectively Pol II and Pol V dependent TLS events (Fig 2B) reinforcing the previous results.

Finally, we tested the effect of the two nucleases on the TLS polymerase Pol IV in the presence of the benzo(a)pyrene (BaP) lesion ((53)). Only deletion of *exoI* in the *recB* deficient strain was able to almost fully recover the TLS level of the parental strain (Fig 2C), thus pointing ExoI as the major nuclease acting in the absence of RecBC.

Since loss of RecBC also affects HDGR mechanism (Fig 1), we measured HDGR level in an *exoI recB* strain in the presence of a G-AAF lesion and observed full recovery of HDGR level

similar to the parental strain (Suppl Fig 3A). Hence, from these data it appeared that the defect in lesion bypass observed in the absence of RecBC can be alleviated by inactivation of ExoI.

RecBC protects the nascent DNA from ExoI exonuclease

ExoI is a Mg^{2+}-dependent exonuclease that rapidly processes ssDNA in the 3'-5' direction (54). It is a highly processive nuclease whose activity is stimulated by SSB protein (55). ExoI plays several roles in DNA metabolism and DNA repair, in particular in mismatch repair, recombination and completion of DNA replication (20, 56–58). Hence, we pursued the characterization of the role of ExoI in lesion bypass. As expected, complementation experiments in the *exoI recB* strain, using a plasmid carrying a functional *exoI* gene under its native promoter, resulted in low levels of Pol II TLS events in the presence of a G-AAF lesion similar to the one observed in a *recB* deficient strain (Fig 2A).

To reinforce our hypothesis that ExoI is the exonuclease preventing efficient lesion bypass in the absence of RecBC, we used the well-described mutant allele *sbcB15* (*exoIA183V*), which is inactivated in the nuclease active site (58). We introduced the point mutation in the *exoI* gene by CRISPR-Cas9 technology in the *recB* deficient strain and measured the level of Pol II TLS in the presence of a G-AAF lesion. We observed full recovery of Pol II TLS, similar to the parental strain (Fig 2A).

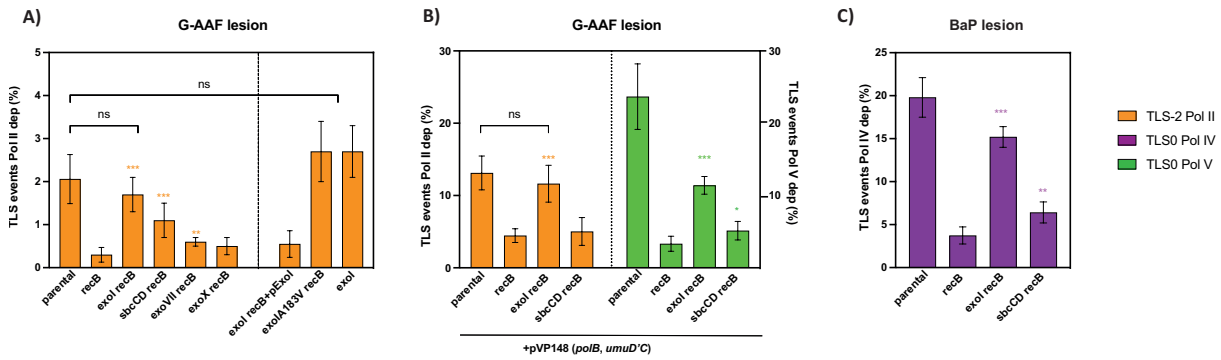


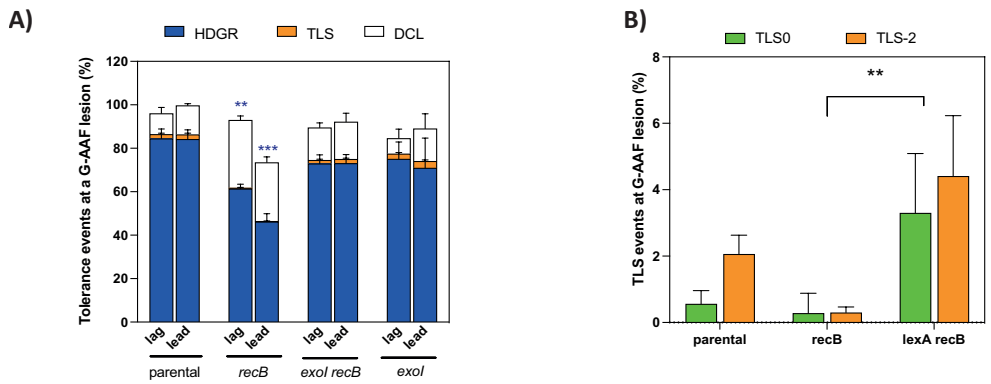
Figure 2. Exonuclease screen. A) The graph represents the percentage of Pol II TLS events in the presence of a G-AAF lesion for several exonuclease mutant strains combined with *recB* deletion. The orange asterisks represent the statistical significance for every exonuclease mutant strain compared to *recB* deficient strain. pExoI is a plasmid expressing *exoI* gene under its own promoter. ExoIA183V is the nuclease dead allele of ExoI (also known as *sbcB15*). **B)** The graph represents the percentage of Pol II and Pol V TLS events in the presence of a G-AAF lesion. All the strains contain the pVP148 plasmid that expresses *polB* and *umuD'C* genes under their native promoters. The orange and green asterisks represent the statistical significance for the mutant strains compared to *recB* deficient strain. **C)** The

graph represents the percentage of Pol IV TLS events in the presence of a BaP lesion. The violet asterisks represent the statistical significance for the mutant strains compared to *recB* deficient strain. The data in every graph represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation. The data are normalized to cell survival. Statistical analyses have been performed with Prism using an unpaired *t*-test: * $p < 0,05$; ** $p < 0,005$; *** $p < 0,0005$.
NB: The data for the parental and *recB* strains in A) and C) panels have been already published in (17).

Taken together these data indicate that in the absence of RecBC, ExoI is the major exonuclease that negatively affects lesion bypass. Given that its exonuclease catalytic activity is required, we surmise that it acts most likely by degrading the nascent DNA that presents a 3' extremity blocked at the lesion site. We wondered whether in the presence of RecBC, ExoI can still access to the 3'-end of the nascent DNA, thus affecting lesion bypass. We inactivated *exoI* gene in the parental strain and measured Pol II TLS events and HDGR in the presence of a G-AAF lesion. No increase neither in Pol II TLS events, nor in HDGR mechanism were observed compared to the parental strain (Fig 2A and Suppl Fig 3A) suggesting that RecBC, when present, efficiently protects the nascent DNA by counteracting the action of ExoI.

ExoI and the TLS polymerases are in competition for the same substrate

Based on these genetic data, we proposed that the RecBC complex protects the nascent DNA blocked at the lesion site from the attack of the ExoI nuclease in order to allow efficient lesion bypass, either by TLS polymerases or by HDGR mechanism. In the absence of RecBC, ExoI can access the 3'-end of the nascent DNA degrading it and affecting lesion bypass. According to this model, in the absence of RecBC, the action of ExoI is most likely in competition with lesion tolerance pathways, in particular with the TLS polymerases since their cognate substrate (the 3'-end) is the same. Therefore, overexpression of the TLS polymerases should partially compensate for the absence of RecBC. Indeed, from the data obtained with a plasmid overexpressing Pol II and the active form of Pol V, we can see that TLS events in a *recB* strain are much higher compared to the *recB* strain that does not overexpress Pol II and Pol V (Fig 2A and 2B). Further confirmation came from testing both Pol II and Pol V TLS levels in a strain deficient for both *recB* and *lexA*: in such strain, constitutive SOS induction leads to an increase in the expression level of TLS polymerases that in turn lead to a 15-fold increase of both TLS events (TLS-2 and TLS0) compared to a *recB* strain (Suppl Fig 3B).



Suppl Figure 3: A) The graph represents the partition of lesion tolerance pathways (*i.e.* HDGR, TLS and DCL) and cell survival in the presence of a G-AAF lesion for *exoI* deletion strains. The lesion was inserted either in the lagging (lag) or in the leading (lead) strand. The data represent the average and standard deviation of at least three independent experiments. No statistically difference for the *exoI* strains (unpaired *t*-test analysis) was observed compared to the parental strain. **B)** The graph represents the Pol II and Pol V TLS events in the presence of a G-AAF lesion for the *lexA recB* deficient strain. The data represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation. The asterisk represents the statistical significance for the *lexA recB* strain compared to *recB* deficient strain for both TLS events.Statistical analyses have been performed with Prism using an unpaired *t*-test: ** $p < 0,005$; *** $p < 0,0005$.

RecBC needs to bind to the DNA to protect the nascent DNA

RecB is a 134 kDa protein composed of two parts, the N-terminal region belonging to the superfamily 1 (SF1) helicase and the C-terminal region containing the nuclease activity (31, 59) and the RecA interaction motif (32, 60). The two regions are connected by a 70 amino acid linker (see Fig 3A). We previously showed that neither the nuclease activity or the RecA loading activity of RecB were required for lesion bypass. Indeed, the *recB*^{D1080A} mutant allele behaved as the parental strain for both TLS and HDGR pathways and the expression of the *recA730* allele, that does not require a mediator to load itself on ssDNA, did not restore the level of lesion tolerance pathways in the absence of RecB (see (17) and Fig 3B). Therefore, we decided to investigate whether the SF1 helicase core of the protein could be involved in the protection of the nascent DNA.

SF1 helicases are composed of a four-subdomain structure (1A, 1B, 2A, 2B) that contains seven conserved motifs (61, 62) (See Figure 3A): motif I contains the Walker A box responsible for binding the triphosphate tail of ATP; motif II is the "DEAD" box motif responsible for Mg+ binding, required for ATP hydrolysis; motif III, IV and VI are involved in ATP hydrolysis and in coupling ATP hydrolysis to DNA translocation (62–64); motif Ia, III and

V were shown to contain residues involved in DNA binding and ssDNA translocation (61, 64, 65).

In *E. coli* the UvrD and Rep helicases are representative of this SF1 family. We performed multiple protein sequence comparison with these two helicases as well as other well studied bacterial SF1 helicases and identified in RecB all the characteristic SF1 motifs and most of the conserved amino acids responsible for the binding of NTPs or DNA (see Fig 3A). We hypothesized that RecBC needs to bind DNA in order to protect from ExoI degradation. Hence, we mutated specific amino acid residues of RecB presumably involved in DNA binding and monitor the effect on lesion bypass. Based on several crystal structure studies, residues in Motif Ia and III seemed to be the most indispensable for ssDNA binding (61, 64, 65), therefore we generated the mutant allele *recB*^{F64A}, in which the phenylalanine 64 of Motif Ia is mutated to alanine, and the mutant allele *recB*$^{YFR\text{-}AAA}$, in which tyrosine 420, phenylalanine 422 and arginine 423 of Motif III have all been mutated to alanine (see Fig 3A). We monitored Pol II TLS events in these mutant alleles in the presence of a G-AAF lesion: TLS decreased to a level similar to the *recB* deficient strain (Fig 3B), indicating that these residues are required for the protective role of RecBC.

The ATPase activity of RecB is also required to protect nascent DNA

Another essential function of the SF1 helicase core is the binding and hydrolysis of ATP, the energy motor for the DNA translocase and helicase activity. Mutagenesis of lysine 29 in Motif I of RecB (RecB^{K29Q}) is known to inactivate the ATPase activity of the protein (38, 66). We tested the role of this mutant allele in DNA damage tolerance: Pol II TLS events halved compared to the parental strain (Fig 3B), suggesting that the ATPase activity is also required. This result is not unexpected since the ATPase activity is also required for the ssDNA binding and for the translocation activity (67). We confirmed these results using the plasmid that overexpresses Pol II in the *recB*^{K29Q} and the *recB*$^{YFR\text{-}AAA}$ strains. In these mutants, despite the increased level of Pol II, the level of TLS-2 events remains low, similar to the *recB* deficient strain expressing the same plasmid (Suppl Fig 4), indicating that RecBC requires to interact with DNA to assure its protective role.

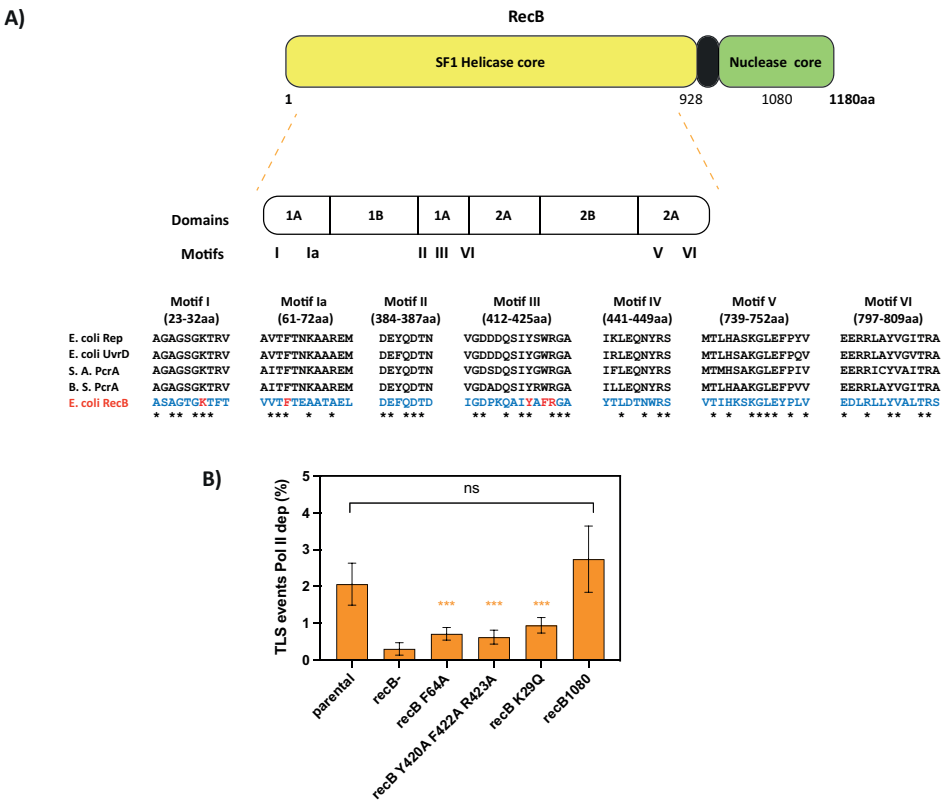
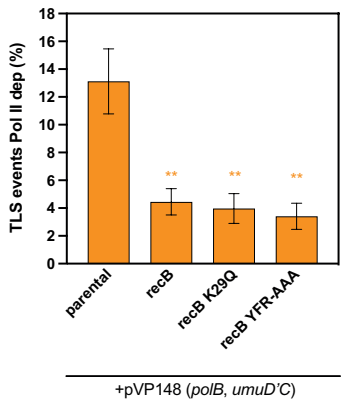


Figure 3. Involvement of RecB domains. A) Domain organization of RecB protein: the N-term SF1 helicase core region (residue 1-928) is linked by a 70 aa linker to the C-term nuclease core (residue 998-1180). The SF1 helicase core is composed of two domains (1 and 2), each divided in two subdomains (A and B) and seven conserved motifs (I, Ia, II, III, IV, V, VI). The aa positions of the *E. coli* motifs are indicated in brackets. S.A stands for *Staphylococcus aureus* and B.S for *Bacillus stearothermophilus*. In red are indicated the amino acid residues that we have mutated by CRISPR-Cas9 technology. **B)** The graph represents the Pol II TLS events in the presence of a G-AAF lesion of several mutant alleles of RecB. The orange asterisks represent the statistical significance compared to parental strain. The data represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation. The data are normalized to cell survival. Statistical analyses have been performed with Prism using an unpaired *t*-test. *** $p < 0,0005$. NB: The data for the parental, *recB* and *recB*^{D1080A} strains have been already published in (17).



Suppl Figure 4: The graph represents the Pol II TLS events in the presence of a G-AAF lesion for some mutant alleles of RecB in the presence of the plasmid expressing Pol II under its own promoter (thus

Pol II expression is increased 3-5 times). The data represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation. The orange asterisks represent the statistical significance compared to parental strain. The data are normalized to cell survival. Statistical analyses have been performed with Prism using an unpaired *t*-test. ** p < 0,005.

RecBC protects the nascent DNA in the presence of a ssDNA gap

We have shown the requirement of RecBC in the presence of strong blocking lesions such as G-AAF and UV lesions (13, 17), when a ssDNA gap is formed downstream the lesion. When replication is stalled by a less blocking lesion, bypass can occur without the need for replication to restart downstream of the lesion: in this situation, no ssDNA gap will form as the lesion will be rapidly bypassed by the replicative polymerase or a TLS polymerase. Therefore, we asked whether in this situation RecBC was still required to protect the nascent DNA. For this, we used three less blocking lesions, the amino fluorene (AF) (68), the hydroxypropyl (1hpG) (69) and the N2-G-furfuryl (70). As we can see in Fig 4, TLS events at these lesions are more frequent than for the strong blocking lesions, such as the G-AAF or the UV photoproducts (12). In the absence of RecBC no significant decrease in lesion bypass was observed for the three lesions (< 1,5-fold) (Fig 4), while we have measured a 7-fold decrease in the absence of RecBC when stronger blocking lesions were used (see (17) and Fig 2). These data indicated that RecBC is especially required in the presence of a persistent ssDNA gap, probably to protect the nascent DNA until the gap is filled in by HDGR or TLS mechanisms post-replicatively.

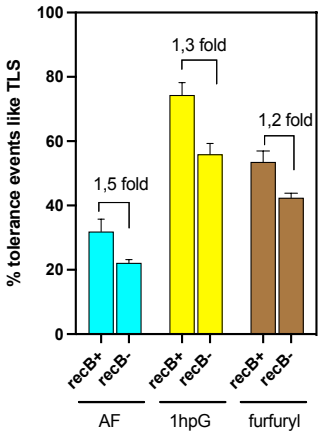


Figure 4: RecBC is required for efficient lesion bypass only in the presence of strong-replication blocking lesions.

The graph represents the tolerance events TLS like in the presence of less blocking lesions due to DNA pol III or to a TLS polymerase bypass. The data represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation.

RecBC complex binds to ssDNA gap in the presence of SSB

In the early 80s, as RecD has not yet been identified, several works studied the activity of RecBC in the presence of circular gapped substrates, that could mimic the DNA intermediates during replication and/or recombination (35, 71), showing a weak exo and endonuclease activity as well as a weak helicase activity for RecBC. While these studies have suggested a role for RecBC in the presence of a gap, they did not investigate whether RecBC could actually bind to a ssDNA gap. From our genetic data we know that the amino acids responsible for the DNA binding are necessary for efficient TLS bypass (Fig 3B) and that RecBC is required in the presence of strong blocking lesions when a ssDNA gap is formed. Therefore, we performed *in vitro* assays to test whether RecBC could bind to a ssDNA gap in order to confirm and support our model.

We constructed two circular gapped DNA substrates using the phage phiX174 (ϕX174), with two different ssDNA gaps of 5.3 kb and 2,5 kb (Fig 5A). In parallel, we expressed and purified the RecBC complex according to (34) (Suppl Fig. 5A). As mentioned before, the RecBC complex was shown to lack exonuclease activity while still preserving a weak helicase activity (44, 67, 72). Our purified RecBC complex was indeed not able to degrade a linear blunt DNA substrate but we observed a weak helicase activity with the same DNA substrate (Suppl Fig 5B and 5C).

We then performed electrophoretic mobility shift assays using our gapped substrates. When using only the RecBC complex, no shift was observed with either substrate (Fig. 5B). However, when we added to the reaction the single-stranded DNA binding (SSB) protein, which covers the ssDNA gap, the RecBC complex produced an additional band shift for both DNA substrates (Fig 5B). The binding of RecBC in the presence of SSB is specific to the ssDNA gap, since no binding was observed with a circular ssDNA plasmid even in the presence of SSB (Suppl Fig 5D).

The acidic tip of SSB is not required for RecBC binding to DNA gaps

To further characterize this cooperative binding of RecBC with SSB, we asked whether there was a direct interaction between RecBC and SSB. Indeed, bacterial SSBs are known to

be a hub for protein recruitment, in particular their C-terminal tail contains several amino acids (also called the "acidic tip") responsible for the binding of several proteins involved in recombination, repair and replication (73–78). We performed mobility shift assay, using a well-described truncated form of SSB (SSBΔ8) that lacks the last 8 aa, thus affecting protein-protein interaction (79), but not the DNA binding capacity (73). In the presence of the SSBΔ8, the RecBC complex was still able to bind to the DNA substrate (Fig 5C), suggesting no direct interaction between the two proteins. It appears therefore that it is more a structural conformation created by the binding of SSB proteins to the ssDNA gap the requirement for RecBC binding. Our data, showing that RecBC cannot bind to a circular ssDNA plasmid even in the presence of SSB (Suppl. Fig 5) seems to support this result, *i.e.* no direct binding between RecBC and SSB. However, we cannot completely rule out the possibility that RecBC does interact with SSB, in particular with its IDL (intrinsically disordered linker) domain since recently it has been shown that this part of the protein might also be involved in the protein-protein interaction (77, 78, 80).

ExoI needs a helicase to access the nascent DNA

We then wanted to assess *in vitro* whether RecBC can prevent degradation of the 3'-end of the gapped substrate due to ExoI activity. ExoI is known to degrade single stranded DNA with high processivity and to dissociate upon encountering with dsDNA (50, 54). In agreement with this, when we tested the activity of ExoI on our gapped DNA substrate, we did not observe any degradation except when we added a flap of more than 6nt (Suppl Fig 5G). Therefore, we hypothesize that ExoI acts on the nascent DNA with the help of a helicase that provides the right substrate for the exonuclease and that most likely RecBC prevents the access of this helicase to the ssDNA gap. In an attempt to identify this putative helicase, we have performed a helicase screen (only non-essential helicases) in the *recB* deficient strain, similar to the one done for the nucleases. None of the tested candidates (*uvrD, dinG, yoaA, helD* genes) restored the level of TLS as the parental strain (Suppl Fig 6). Therefore, without the identification of this helicase and its addition to the reaction, we were not able to assess *in vitro* whether RecBC protects against DNA degradation. Nevertheless, we were able to show that RecBC prevents ExoI degradation when it binds to an oligo (Suppl Fig 5E), suggesting that this could also occur when RecBC binds to a ssDNA gap.

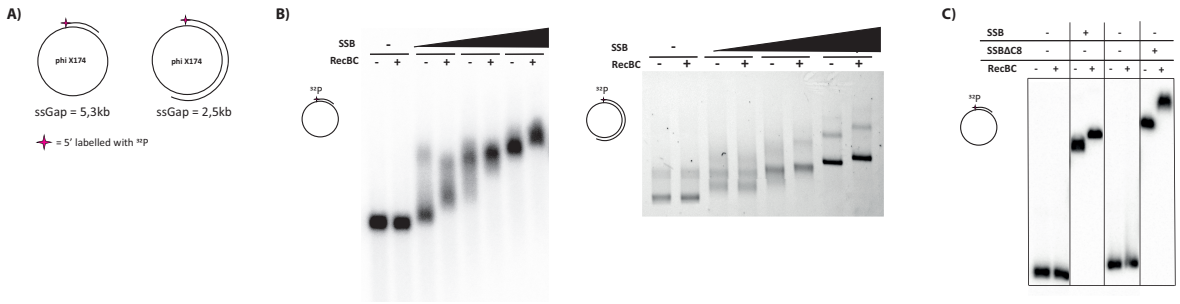
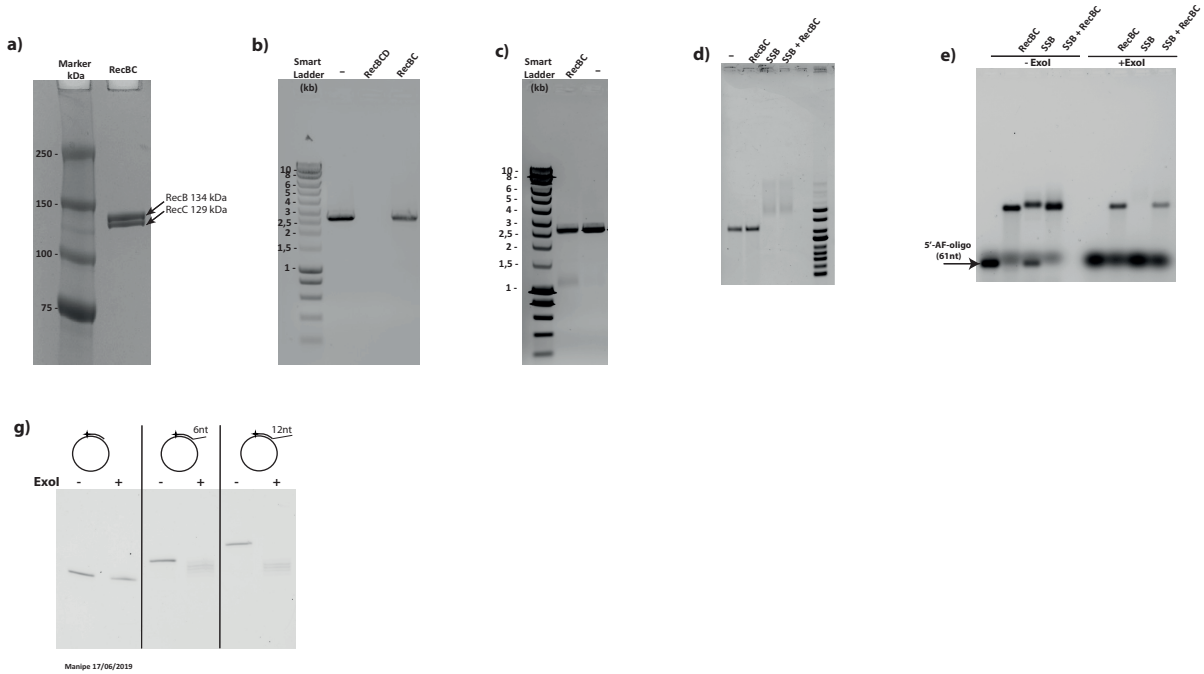


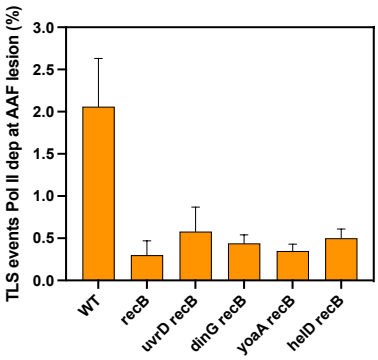
Figure 5: RecBC complex binds to a ssDNA gap in the presence of SSB

A) DNA substrates used for the electrophoretic mobility shift assays. The circular ssDNA of the phage ϕX174 was used to obtained a 5.3kb ssDNA gap or a 2.5kb ssDNA gap. The 5'of the ds-ss junction of the substrates have been radiolabeled with ^{32}P. **B)** Mobility shift assays on agarose gel using both DNA substrates in the presence of RecBC complex and with (or without) increased concentrations of SSB (see Material and Methods for more details). In the absence of SSB, no band shift is observed for RecBC alone. **C)** Mobility shift assay on agarose gel using the 5.3kb DNA gap in the presence of the truncated form of SSB (SSBΔ8).



Suppl Figure 5: A) Protein gel Coomassie stained showing the purified RecB and RecC proteins. **B)** Nuclease assay using a linear dsDNA substrate and comparing the activity of RecBC complex versus the RecBCD complex (Provided by NEB). **C)** Helicase assay for RecBC complex using a linear dsDNA substrate. The difference here from the nuclease assay is the concentration of Mg and the temperature of the reaction (for more details, see Supplementary M&M section). **D)** Mobility shift assay on agarose gel using a circular ssDNA plasmid (ϕX174) in the presence of RecBC, SSB or the two proteins together. **E)** On the left side of the gel, mobility shift assay using a 61nt oligo. RecBC can bind without SSB and no further shift is observed in the presence of SSB. On the right side of the gel, same mobility shift assay but adding ExoI to the reaction. The presence of RecBC bound to the oligo protects against ExoI

degradation. **G)** Nuclease assay of ExoI using a gapped substrate and gapped substrates containing a flap of 6 or 12 nt.



Suppl Fig 6. The graph represents the Pol II TLS events in the presence of a G-AAF lesion for several helicase genes in the *recB* deficient strain. The data represent the average and standard deviation of more than four independent experiments obtained in the lagging or in the leading orientation.

DISCUSSION

This study unveils a cellular role for the RecBC complex, without the RecD subunit, in protecting the nascent DNA during lesion encounter. This mechanism allows efficient lesion bypass in the presence of a single-stranded DNA gap. Indeed, in the absence of RecBC lesion bypass is severely compromised, because degradation of the nascent DNA most likely prevents the recruitment of the TLS polymerases and also affects the efficiency of the HDGR mechanism. While we have recently shown that the ssDNA gap needs to be enlarged on its 5' end by the action of the nuclease RecJ and helicase RecQ to perform efficient HDGR (16), the present study shows that the 3' end of the gap needs to be protected from nucleolytic degradation operated by the exonuclease ExoI in order to perform an efficient lesion bypass and complete the replication of a damaged DNA.

Based on our *in vivo* and *in vitro* results, we proposed a model (Fig 6) in which RecBC is required to protect the nascent DNA only when a ssDNA gap is formed and persist. The ssDNA gap is immediately covered by the SSB protein that protects the ssDNA against nuclease cleavage and orchestrates the recruitment of several proteins for genome integrity maintenance. Once SSB covers the ssDNA gap, RecBC complex can bind to the ss-dsDNA junction to protect the nascent DNA from the action of the nuclease ExoI (and a still to identify helicase) and allow an efficient lesion bypass. This situation is typical of a strong roadblock when replication needs to restart downstream in order to continue replication, creating a persistent ssDNA gap that will be later filled in by one of the two lesion tolerance pathways. On the other hand, when replication is stalled by a less blocking lesion that can be easily bypassed by either the replicative polymerase or a TLS polymerase, the ssDNA gap created by the DNA unwinding is only transient and RecBC protection is not required.

SSB is a constitutive component of the replisome, namely associating with the ssDNA on the lagging strand during Okazaki fragment formation or ssDNA created during the DNA helicase unwinding. However, the SSB binding lifetime to these "physiological" ssDNA gaps and the SSB binding lifetime to the ssDNA gaps formed upon lesion encountering are probably different, as recently suggested by Loparo's group (81). They also suggested that the mobility change in SSB molecules upon replication perturbation could act as a molecular signal to enrich proteins at the lesion site, as it is the case for Pol IV TLS polymerase (70, 81). Hence, this difference in SSB dynamics provides an explanation why RecBC is specifically recruited to protect the nascent DNA of the ssDNA gap formed in the presence of strong blocking lesions

and not in the presence of a less blocking lesion, as well as why it is not recruited to the ssDNA gaps formed during Okazaki fragment.

While RecBC protects the 3'-end of the ssDNA gap, the gap is enlarged on the 5'-end by the action of the nuclease RecJ and the helicase RecQ before the mediator proteins RecF-O-R load RecA to form the recombinogenic filament as we have recently proposed (16). It is possible that the RecBC protection is required until the RecA filament is formed and it can undergo homology search and strand invasion. From this moment on, the nascent DNA does not need any more protection.

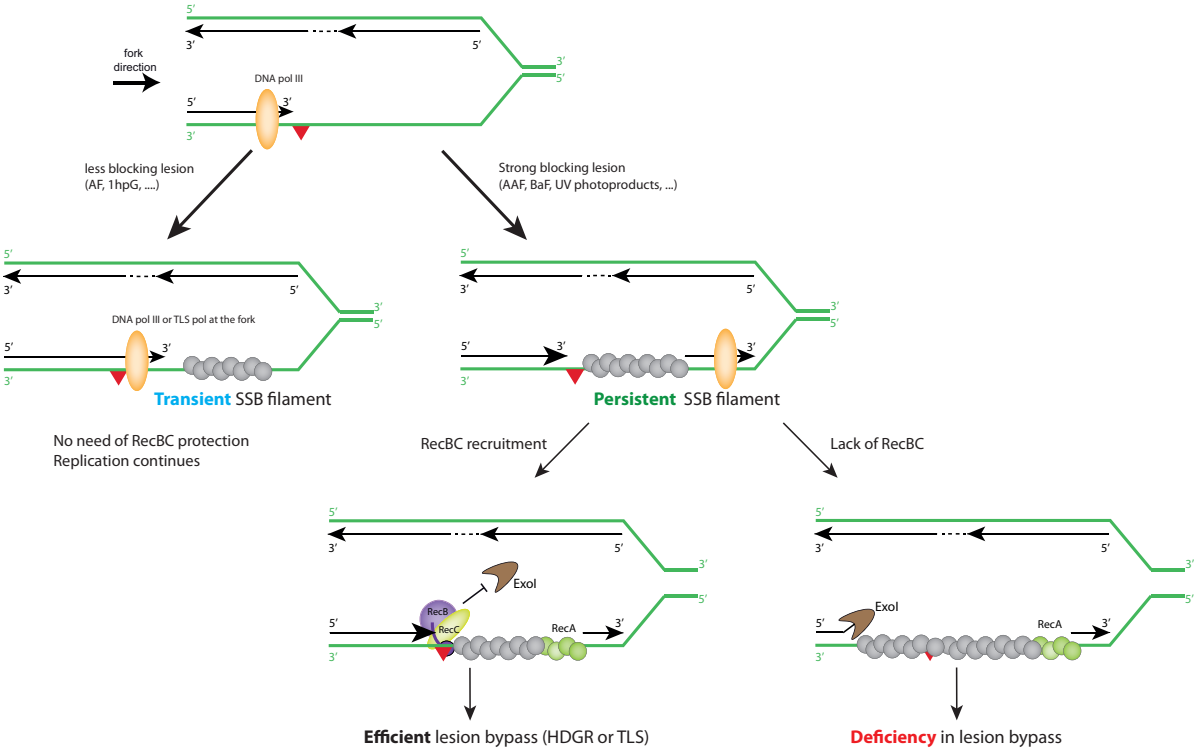


Figure 6: Model of RecBC complex protecting the nascent DNA in the presence of a ssDNA gap. The replicative DNA polymerase is temporarily blocked at a DNA lesion : i) when the lesion can be easily bypassed by the replicative DNA polymerase itself or by a TLS polymerase at the fork, the SSB-covered ssDNA formed by the DNA unwinding is transient and the RecBC complex is not recruited at the lesion site; ii) in the presence of a strong blocking lesion, replication restarts downstream creating a persistent SSB-covered ssDNA gap: RecBC is recruited at the ds-ssDNA junction to protect the nascent DNA against nuclease attack. This will allow efficient lesion bypass by one of the two lesion tolerance pathways. In the absence of RecBC, ExoI can access the 3-end of the nascent DNA (with the help of a helicase) degrading the nascent DNA. This will impact lesion bypass, in particular the action of the TLS polymerases, but also the HDGR mechanism.

In the absence of RecBC, in a sort of "pathological condition", the nascent DNA is deprotected and we proposed that ExoI can access the nascent DNA with the help of a still to

be identified helicase and degrade it, thus strongly inhibiting TLS polymerases access/activity since ExoI and the TLS polymerases are in competition for the same substrate (the 3'-end). In line with this, overexpression of the TLS polymerases can partially outcompete with the action of ExoI. Degradation of the nascent DNA also affects HDGR leading to an increase in damage chromatid loss events (DCL, Fig 1). However, high levels of HDGR still persist in the absence of RecBC so either not all the nascent DNA is degraded by ExoI or the action of ExoI is also kinetically in competition with the RecA filament formation. Indeed, it is likely that once the recombinogenic filament is formed, it will engage in homology search and strand invasion and ExoI will not be able to access the nascent DNA. This is corroborated by the phenotype of the double mutant *recF recB* in a previous article (13) where we observed a strong loss of HDGR and cell survival (similar to a *recA* deficient strain). Delaying the formation of the RecA filament (in the absence of its mediator RecF) in addition to the loss of RecBC will favor ExoI activity, thus degradation of the nascent DNA will strongly impact lesion bypass and replication.

In the last decade, growing evidence indicate that the nascent DNA must be protected against uncontrolled and excessive nucleolytic degradation, failure to do so might severely compromise the completion of DNA and the stability of the genome (82, 83). Surprisingly, proteins usually involved in homologous recombination pathway, such as RAD51 and BRCA1/2 in humans, came to the fore as guardians of the nascent DNA against the attack of specific nucleases (48, 84, 85). This function is distinct from their canonical function in homologous recombination. Similarly, in this study we have shown that the RecBC complex, that is part of the RecBCD complex involved in homologous recombination repair in *E. coli* and is one of the mediators of RecA, plays a new and unexpected role in lesion bypass, independent of its canonical catalytic activities. This new finding indicates once more how the mechanisms of DNA replication are evolutionary conserved in all living organisms. It will be interesting to investigate in human cells whether lesion bypass may also be compromised in cells deficient for the so-called fork protection when a ssDNA gap is formed.

MATERIAL AND METHODS

Bacterial strains and growth conditions

All *E. coli* strains used in this work are listed in Supplementary Table 1. They are derivatives of strains FBG151 and FBG152 (12), that carry a plasmid that allows the expression of the *int–xis* genes after IPTG induction. Strains were grown on solid and liquid Lysogeny Broth (LB) medium. Gene disruptions were achieved by the one-step PCR method (86) or by P1 transduction using the Keio collection (87). Following the site-specific recombination reaction, the lesion is located either in the lagging strand (FBG151 derived strains) or in the leading strand (FBG152 derived strains). The mutant alleles of *exoI* (*exoI*^{A183V}) and *recB* (*recB*^{K29Q}, *recB*^{F64A}, *recB*^{Y420AF422AR423A}) have been obtained by CRISPR-Cas9 technology adapted for *E. coli* according to (88). These strains have been verified by sequencing (the entire modified gene). Antibiotics were used at the following concentrations: ampicillin 50 or 100 µg/ml; tetracycline 10 µg/ml, kanamycin 100 µg/ml, chloramphenicol 30 µg/ml. When necessary, IPTG and X-Gal were added to the medium at 0.2mM and 80 µg/ml, respectively.

Plasmids

pVP135 expresses the integrase and excisionase (*int–xis*) genes from phage lambda under the control of a *trc* promoter that has been weakened by mutations in the -35 and the -10 region. Transcription from Ptrc is regulated by the *lac* repressor, supplied by a copy of *lacI*^{q} on the plasmid. The vector has been modified as previously described (12).

pVP146 is derived from pACYC184 plasmid where the chloramphenicol resistance gene has been deleted. This vector, which carries only the tetracycline resistance gene, serves as an internal control for transformation efficiency.

pVP141-144 and pGP9 are derived from pLDR9-attL-lacZ as described in (12). pLL1 and pLL7 are derived from pVP141 as previously described (13). pAP1, pAP2 and pGP22 are derived from pVP143. All these plasmid vectors contain the following characteristics: the ampicillin resistance gene, the R6K replication origin that allows plasmid replication only if the recipient strain carries the *pir* gene, and the 5' end of the *lacZ* gene in fusion with the *attL* site-specific recombination site of phage lambda. The P'3 site of *att*L has been mutated (AATCATTAT to AATTATTAT) to avoid the excision of the plasmid once integrated. These plasmids are produced in strain EC100D pir-116 (from Epicentre Biotechnologies, cat# EC6P0950H) in which the pir-116 allele supports higher copy number of R6K origin plasmids. Vectors carrying a

single lesion for integration were constructed as previously described following the gap-duplex method (12). A 15-mer oligonucleotide 5'-ATCACCGGCGCCACA-3' containing or not a single G-AAF adduct (underlined) in the *Nar*I site was inserted into the gapped-duplex pLL1/7 (to measure HDGR) or into the gapped-duplexes pVP141-142 or pVP143-144 to score respectively for TLS0 Pol V-dependent and for TLS-2 Pol II-dependent. A 13-mer oligonucleotide, 5'-GAAGACCTGCAGG, containing no lesion or a dG-BaP(-) lesion (underlined) was inserted into the gapped-duplex pVP143/pGP9 to measure Pol IV TLS events. A 14-mer oligonucleotide 5'-ATACCCGGGACATC-3' containing no lesion or a single G-AF or a 1hpG adduct (underlined) was inserted into the gapped-duplex pAP1-AP2 to measure TLS like events. A 20-mer oligonucleotide 5'-CTACCTGTGGACGGCTGCGA-3' containing or not the N^2-furfuryl adduct was inserted into the gapped duplex pAP1-pGP22 to measure TLS like events. All these new batches of plasmid constructions are carefully quantified and validated by integration in the parental strain.

pVP148 plasmid expresses both *polB* and *umuD'C* loci under their own promoters. It is derived from pRW134 (89), a pGB2 vector that carries *umuD'C*, in which the spectinomycin resistance gene was replaced by the chloramphenicol resistance gene. The *polB* gene was added by subcloning a SalI-BamHI fragment from pYG787 (90). UmuD' is the cleaved version of UmuD that forms the heterotrimeric complex $(UmuD')_2UmuC$, *i.e.*, the active form of Pol V (51).

pLL70 is a plasmid expressing *exoI* gene under its own promoter. It was obtained by removing the *umuDC* gene and its promoter from pVP145 by PstI/BamHI digestion and cloning *exoI* gene+promoter by PstI/BamHI. pVP145 is derived from pRW134 (89), in which the spectinomycin resistance gene was replaced by the chloramphenicol resistance gene.

Integration protocol: monitoring HDGR and TLS events

The protocol for lesion integration assay is described in details in (17, 45). Cells were diluted and plated before the first cell division using the automatic serial diluter and plater EasySpiral Dilute (Interscience) and were counted using the Scan 1200 automatic colony counter (Interscience).

For every integration experiment, we used the lesion versus non-lesion plasmid constructions, each plasmid solution containing an equal amount of pVP146 plasmid as internal control. Following the integration of the pLL1/7 vector (AAF lesion), sectored blue/white colonies represent HDGR events. Following integration of the vectors pVP141/142 (TLS0 for G-AAF),

pVP143/144 (TLS-2 for G-AAF), pVP143/pGP9 (BaP lesion), pAP1-AP2 (G-AF or 1hpG lesions), pAP1-pGP22 (N^2-furfuryl lesion) sectored blue/white colonies represent TLS events. The relative integration efficiencies of lesion-carrying vectors compared with their lesion-free homologues, normalized by the transformation efficiency of pVP146 plasmid in the same electroporation experiment, allow the overall rate of lesion tolerance to be measured (which corresponds to the cell survival in the presence of a single lesion). In the parental strain one lesion gives about 100% cell survival. For the G-AAF lesion we combine the results obtained with the different plasmids to measure HDGR and TLS events. Briefly, the mean of the absolute values (blue colonies/total colonies) of HDGR and TLS events were multiplied by the mean of the cell survival obtained with all the plasmids construction for a given lesion. Indeed, whether TLS or HDGR was measured, cell survival will not change for a given lesion. The value of DCL was calculated by subtracting the HDGR and TLS values to the total cell survival.

Tolerance events (Y axis) represent the percentage of cells able to survive in presence of the integrated lesion compared to the lesion-free control. The data in every graph represent the average and standard deviation of at least three independent experiments of a lesion inserted in the leading (or in the lagging) orientation. Statistical analyses were done using GraphPad Prism applying an unpaired *t*-test.

Sequence alignment

We used Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) for multiple protein sequence alignment to identify the seven conserved motifs specific of SF1 helicases in RecB. For the alignment, we used the Rep and UvrD helicase from *E. coli*, the PcrA helicase from *Staphylococcus aureus* and *Bacillus stearothermophilus* according to (61, 62).

RecBC protein expression and purification

The protocol for RecBC expression and purification has been adapted from (34). Briefly, *E. coli* RecBC complex was purified from BL21 DE3 Δ*recBD* strain harboring B13 (pETduet-His_6-TEVsite-recB, Amp^R) and B3 (pRSFduet-recC, Kan^R) plasmids. The cells were induced with 1mM IPTG at mid-log phase (0.6 OD_{600}) and grown for 4h at 37°C. The lysate was purified on HisTrap HP Ni-Sepharose (Cytiva), then dialyzed overnight with TEV protease to cleave the His-tag. The His-tag was removed by a second step on HisTrap HP column. The complex was further purified using HiTrap Heparin-Sepharose (Cytiva), then HiTrap Q FF Sepharose (Cytiva) before

continuing with MonoQ (Cytiva) anion exchange chromatography. The protein was dialyzed overnight in 20 mM Tris-HCl pH8, 1mM EDTA pH 8, 1mM DTT, 50mM NaCl, 10% glycerol and flash-frozen.

Preparation of the gapped DNA substrates

Plasmid with a 5,3 kb ssDNA gap: 3 pmol of a 50nt oligonucleotide (VP683, TCGAGCTGCGCAAGGATAGGTCGAATTTTCTCATTTTCCGCCAGCAGTCC) was 5' radiolabelled using 3,3 pmol of γ-^{32}P-ATP (Perkin Elmer) and 10 units of PNK (NEB) in a total volume of 25µl PNK buffer 1X. The reaction was incubated during 30 minutes at 37°C and inactivated during 20 minutes at 65°C. Then it was purified using a MicroSpin G-25 column (Cytiva). The oligonucleotides (60nM) were hybridized to φX174 ssDNA plasmids (5.3 kb, NEB) (20nM) in 50 µl buffer containing 10 mM Tris-HCl pH8, 1mM EDTA pH8 and 50mM NaCl. The reaction was incubated in water at 75°C and let cool down to room temperature. Substrates were checked on 0,8% agarose gel.

Plasmid with a 2,5 kb ssDNA gap: 10 µM VP 1394 oligonucleotides (ACGATTAACCCTGATACCAA) were phosphorylated with 1mM ATP (Sigma) and 10 units PNK (NEB) in 50 µl PNK buffer 1X (30' at 37°C, inactivation 20' at 65°C). A 2,8 kb dsDNA fragment was obtained by PCR with Q5 Polymerase (NEB) using phosphorylated VP 1394, VP 1361 (ACGCCCTGCATACGAAAAGA) and φX174 as template. PCR products were digested by ExoI (NEB) to remove excess of oligos and purified using Qiagen PCR purification kit. The phosphorylated DNA strand was then specifically digested with lambda exonuclease (NEB); the ssDNA strands obtained were checked on 0,8% agarose gel. They were 5'-radiolabelled using γ-^{32}P-ATP and purified using a MicroSpin G-25 column. Then they were hybridized to φX174 ssDNA plasmids (ratio 1 :1) in 50 µl buffer containing 10 mM Tris-HCl pH8 and 50mM NaCl. The reaction was incubated in water at 80°C and let cool down to room temperature. Substrates were checked on 0,8% agarose gel.

Electrophoretic mobility shift assays

RecBC complex was expressed and purified in our laboratory as described above, while SSB protein was provided by BioAcademia, and the truncated SSB ΔC8 protein was kindly provided by Michael Cox.

When using the 5,3 kb ssDNA gap substrate 4 fmol of radiolabelled DNA substrates were first incubated for 20' at RT with increasing amounts of SSB (ratio protein:DNA 0-32-65-130:1) in 10 μl binding buffer (20 mM Tris-HCl pH8, 100 μg/ml BSA-Ac, 1mM DTT and 1mM Mg-Ac). Then RecBC (or RecBC buffer as a negative control) was added to the reaction (ratio protein:DNA 400:1) and incubated for 20' at RT. 1X loading dye was added and the samples were loaded on 0,6% agarose gel, running at 4°C. The gel was dried for 1h30 at 60°C, exposed overnight to a Fuji screen and scanned with a Typhoon (Cytiva).

The same reaction conditions were used in Fig 5C except for protein:DNA ratios for SSB and SSB ΔC8 (ratios 0-195:1).

When using the 2,5 kb ssDNA gap substrate 15 fmol radiolabelled DNA substrates were first incubated for 20' at RT with SSB (ratios 0-87-173-346:1) in 12 μl binding buffer. Then RecBC (or RecBC buffer as a negative control) was added to the reaction (ratio 428 :1) and incubated for 20' at RT. 1X loading dye was added and the samples were loaded on 0,6% agarose gel, running at 4°C. The gel was dried for 1h30 at 60°C, exposed overnight to a Fuji screen and scanned with a Typhoon (Cytiva).

SUPPLEMENTARY MATERIAL AND METHODS

Nuclease assay

A linearized plasmid (2594 bp) was incubated 30' at 37°C in 50mM K-Ac, 20 mM Tris-Ac, 10mM Mg-Ac, 1mM DTT, 1mM ATP with RecBC or RecBCD (ratio protein:DNA 5:1). The samples were loaded on 1% agarose gel with ethidium bromide and visualized with Chemidoc MP (Biorad). RecBCD was provided by NEB.

Helicase assay

A linearized plasmid (2594 bp) was incubated 30' at 25°C in 50mM K-Ac, 20 mM Tris-Ac, 1mM Mg-Ac, 1mM DTT, 1mM ATP, 0,36μM SSB with RecBC (ratio protein:DNA 5:1). The samples were loaded on 1% agarose gel with ethidium bromide and visualized with Chemidoc MP (Biorad).

Electrophoretic mobility shift assays

<u>φX174 ssDNA plasmid :</u>

30 fmol φX174 plasmid were first incubated for 20' at RT with SSB (ratios 0-173:1) in 10 µl binding buffer (20 mM Tris-HCl pH8, 100 µg/ml BSA-Ac, 1mM DTT and 1mM Mg-Ac). Then RecBC (or RecBC buffer as a negative control) was added to the reaction (ratio 53:1) and incubated for 20' at RT. The samples were loaded on 0,6% agarose gel, running at 4°C. The gel was stained in ethidium bromide and visualized with Chemidoc MP (Biorad).

Fluorescent oligonucleotide :

200 fmol of the fluorescent oligonucleotide 5'-AF647-TCGAGCTGCGCAAGGATAGGTCGAATTTTCTCATTTTCCGCCAGCAGTCCACTTCGATTTA were incubated for 20' at RT with SSB (ratio 2:1) and/or RecBC (ratio 16:1) in 20 µl binding buffer. 10 µl of each reaction was treated with ExoI (NEB, ratio 5:1) during 15 minutes at 37°C. The samples were loaded on 1,5% agarose gel and visualized with Chemidoc MP (Biorad).

ExoI nuclease assay

3 different oligonucleotides 5'-labelled with FAM (VP 770, VP 1332 and VP 1334) were hybridized to φX174 ssDNA plasmids to create gapped-plasmids without flap, with a 6nt flap and with a 12 nt flap, respectively. They were digested by ExoI (NEB) during 30' at 37°C (protein: DNA ratio 5,7:1). Reactions were stopped by addition of formamide Dye. The samples were loaded on denaturing 15% acrylamide gel to visualize the fluorescent oligonucleotides (Chemidoc MP).

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CONFLICT OF INTEREST

none

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