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Review

DNA helicase-SSB interactions critical to the regression and restart of stalled DNA replication forks in *Escherichia coli.*

Piero R. Bianco [1,*]

[1] University at Buffalo, SUNY; pbianco@buffalo.edu
* Correspondence: pbianco@buffalo.edu; Tel.: (716) 829-2599

Abstract: In *E. coli* DNA replication forks stall on average once per cell cycle. When this occurs, replisome components disengage from the DNA, exposing an intact, or nearly intact fork. Consequently, the fork structure must be regressed away from the initial impediment so repair can occur. Regression is catalyzed by the powerful, monomeric DNA helicase, RecG. During this reaction, the enzyme couples unwinding of fork arms to rewinding of duplex DNA resulting in the formation of a Holliday junction. RecG works against large opposing forces enabling it to clear the fork of bound proteins. Following subsequent processing of the extruded junction, the PriA helicase mediates reloading of the replicative helicase DnaB leading to the resumption of DNA replication. The single-strand binding protein (SSB) plays a key role in mediating PriA and RecG functions at forks. It binds to each enzyme via linker/OB-fold interactions and controls fork loading sites in a substrate-dependent manner that involves helicase remodeling. Finally, it is displaced by RecG during fork regression. The intimate and dynamic SSB-helicase interactions play key roles in ensuring fork regression and DNA replication restart.

Keywords: RecG; SSB; Stalled DNA replication fork; DNA repair; DNA replication; helicase; atomic force microscopy; OB-fold; SH3 domain; PXXP motif

1. Introduction

The accurate and faithful duplication of the genome relies on the DNA replication, repair and genetic recombination machinery working closely together [4-8]. This intricate interplay is required as the advancing replisomes frequently encounter roadblocks that have the potential to stall or collapse replication forks [9, 10]. In *E.coli*, each fork is thought to stall and require restarting at least once per cell cycle. The types of impediments to fork progression that exist include proteins bound to the DNA ahead of the replication fork, non-coding lesions in the template DNA, unusual secondary structures that arise in the DNA and either single- or double-strand breaks [6, 12-16]. Each of these roadblocks to DNA replication likely utilizes different subsets of repair enzymes and this is highlighted by the varied recombination and repair gene requirements for dealing with exposure to different types of DNA damaging agents [13, 19-23]. For example, when the replisome encounters a nick in the leading strand, the structure of the fork will collapse and require the processing of the nascent double-strand break by RecBCD followed by strand invasion catalyzed by RecA. This results in the formation of a displacement loop that is then used to reload the replicative helicase DnaB leading to the restart of DNA replication [21].

In contrast, when replisomes encounter an impediment such as a dsDNA crosslink or bound proteins such as RNA polymerase or mutant methyltransferases, a stalled DNA replication fork is

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formed [27]. A stalled fork can be directly restarted or reversed (regressed) (Figure 1 and [10, 13, 19, 28-32]). Consequently, regression occurs in a direction opposite to that of replication, with the fork actively moved away from the site of damage to a region where the nascent, replicated genome is undamaged. This enables the repair machinery to have access to the impediment and facilitate repair. This process is analogous to the clearing of train tracks following a collision and possible derailment.

Which replisome proteins are disengaged from the DNA? At a minimum, this must be DnaB because of the requirement for the restart DNA helicase, PriA [33-35]. However, due to the ability of polymerases to rapidly turn over, it is conceivable that these components, as well as others, might disengage as well [36, 37]. However, protein disengagement may not always occur as recent work from the Marians group has shown [38]. In this study, they demonstrated that the replisome remains stably associated with the fork after a collision with a leading-strand template lesion. Leading-strand DNA synthesis was then reinitiated downstream of the damage in a reaction that is independent of any of the known replication-restart proteins.

If one or more components of the replisome disengage from the DNA then replication cannot progress and the fork becomes stalled. Regression of the stalled DNA replication fork likely occurs (Figure 1A). This can in principle be spontaneous as shown by the Cozzarelli group [39, 40]. It was also proposed to be catalyzed by several proteins including the recombinase RecA, the DNA helicase RecG and the resolvase RuvAB [41-46]. However, the evidence overwhelmingly demonstrates that RecG catalyzes fork regression as explained below.

RecA is unlikely to be involved in fork regression where either the leading or lagging strands contain exposed ssDNA. This follows because RecA is inhibited by SSB. However, if DNA damage is present in the parental duplex DNA ahead of the fork, RecA could nucleate nucleoprotein filaments at the site of damage, to which it binds with enhanced affinity relative to undamaged DNA. Filament extension from the site of damage towards the fork could in principle move the fork in the regression direction as the leading strand arm is encompassed by the growing filament.

For many years a conundrum existed in the fork regression field as it was thought that the branch-specific DNA helicases RecG or RuvAB could each catalyze this reaction. This conundrum was resolved using a combination of bulk-phase biochemical and single-molecule approaches. Studies show that RecG outcompetes an 80-fold excess of RuvAB at forks; that RecG is assisted in fork regression by the single-strand DNA binding protein (SSB) whereas RuvAB is inhibited; that SSB loads RecG onto forks and in the process remodels the helicase and, once bound to the fork, RecG catalyzes an efficient regression reaction [24, 47-49]. Here, the unique attributes of the specialized DNA helicase RecG come into play: the enzyme couples DNA unwinding to rewinding to generate sufficient force so that obstacles bound to either the leading or lagging strand arms are readily displaced [1].

Once the fork has been regressed and repair of the impediment taken place, the PriA DNA helicase takes center stage [50]. Its primary role to mediate loading of the replicative helicase, DnaB onto the DNA resulting in the restart of replication. While PriA can bind to forks on its own, binding is mediated by SSB, with the helicase being remodeled in the process similar to what is observed for RecG [3, 51, 52].

2. Fork regression defined

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The reaction known as fork reversal or fork regression is a unique reaction that requires a specialized DNA helicase (Figure 1A and see Section 3a). In this reaction, the fork is moved away from the impediment in a direction opposite to that of replisome movement, while simultaneously producing DNA structure(s) which, upon further processing result in the reloading of the replisome. Upon close examination of the movement of the DNA junction, it is immediately apparent that both DNA unwinding and duplex rewinding must occur concurrently (Figure 1B). That is to say, the nascent leading and lagging arms of the fork must be unwound, and the duplex parental DNA rewound. Also, and if the nascent arms contain sufficient regions of complementary dsDNA, an additional region of duplex rewinding will be observed, and this is extruded ahead of the regressing fork. When viewed in this manner, the resulting structure resembled the foot of chicken which has three toes. Not surprisingly, this intermediate is known as the "chicken foot intermediate" (Fig. 1A and [41]). Simply by manipulating the arms of the regressed fork to the vertical position and by the

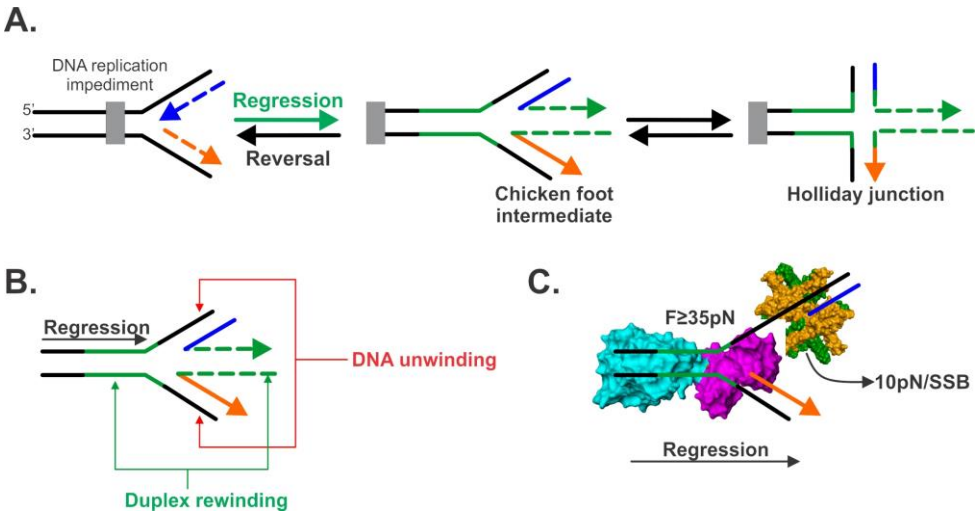

Figure 1. Fork regression. (A). A schematic of the fork regression reaction is presented in the top panel. The fork is shown impeded and the nascent leading (blue) and lagging (orange) strands indicated. Fork regression (green arrow) results in rightward movement of the fork, away from the site of the replication impediment, concomitant with the extrusion of a duplex region resulting from the reannealing of the nascent leading and lagging strands. The resulting DNA structure has been termed the "chicken foot" and is structurally similar to a Holliday junction. Fork reversal results in leftward movement of the fork following regression and subsequent repair. (B). Fork regression requires that DNA unwinding be coupled to duplex rewinding. (C). The RecG DNA helicase is shown in the process of fork regression where it couples DNA unwinding to duplex rewinding and displacement of the SSB protein [42, 62]. RecG and SSB are represented as Connolly surfaces. The wedge domain of RecG is colored purple and the helicase domains in cyan. SSB monomers are colored orange and green.

inclusion of the parental duplex in the picture, it is plain to see that the resulting structure is also a Holliday junction, the primary substrate for the RuvAB resolvase [53]. Formation of the Holliday junction intermediate is central to many models proposed for fork rescue.

In addition to being able to both unwind and rewind DNA strands, the enzyme catalyzing fork regression must be able to clear the DNA near the fork of bound protein obstacles (Fig. 1C). Included

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here are proteins bound to ssDNA gaps such as SSB and which requires 10pN of force for displacement, and proteins that had bound to nascent duplex arms behind the advancing fork such as repressors or even nucleoid-associated proteins. As demonstrated below, a single, monomeric enzyme, the RecG DNA helicase, possesses all of the activities alluded to above and can work against forces >35pN of force during regression.

3. The protein players

a. RecG – the regression beast

RecG protein was identified as a mutation that mildly affected recombination and survival following UV-irradiation [54]. Subsequent studies demonstrated that it participates in all three pathways of recombination and that it has an overlapping function with the products of the *ruvA* and *ruvB* genes [55, 56]. Purified RecG possesses ATPase and DNA helicase activities [55, 57]. It has been classified as a member of the SF2 DNA helicases and nucleic acid translocases [58].

In vitro, RecG unwinds DNA in a 3′ → 5′ direction [59, 60]. It is active as a monomer on forks with either single-stranded or duplex arms as well as Holliday junctions [47]. RecG processes stalled replication fork substrates into structures that can be acted upon by additional members of the recombination machinery [1, 61-64]. In addition to processing branched DNA structures *in vitro*, RecG exhibits significant ATPase activity on negatively supercoiled DNA, single-stranded DNA (ssDNA), and SSB-coated ssDNA [48, 65]. This suggests different ways for RecG to access stalled replication forks, dictated by the types of DNA that might be available in the vicinity of the fork. For example, the strong preference that the enzyme exhibits for negatively supercoiled DNA, suggests that DNA must first be converted from positively to negatively supercoiled for RecG to function [65]. It is known that DNA replication produces positively supercoiled DNA ahead of the advancing replisome [39, 66]. Therefore, once the fork stalls and if the replisome remains bound, DNA gyrase can convert the positively supercoiled DNA into negatively supercoiled. Once the DNA is in this form, RecG catalyzes fork regression efficiently [67].

The stimulation of ATPase activity on SSB-coated M13 ssDNA is intriguing and it was subsequently shown that a species-specific, protein-protein interaction between RecG and SSB was required [2, 48, 51]. This interaction is mediated through the linker domain of SSB and the OB-fold in RecG [68]. SSB-RecG binding is key to helicase function at a stalled fork since the enzyme can be directly loaded onto the DNA in the vicinity of single-stranded regions and is consistent with the role of SSB in targeting repair helicases to active forks *in vivo* [24, 48, 69].

The crystal structure of the enzyme bound to a model fork substrate shows how RecG processes a fork [64]. The structure available is of the *T. maritima* protein and, except for the N-terminal extension whose function is unknown, is very similar to that of *E.coli* RecG [70, 71]. Not surprisingly, homology models of *E.coli* RecG can be built using the *T. maritima* structure (Fig. 2a and [71, 72]). The enzyme is divided into two general domains, highlighted in different colors. Domain I comprises the N-terminal half of the protein and contains the wedge domain which includes the oligonucleotide-oligosaccharide binding fold (Fig. 2). The wedge domain is essential for specific binding to branched DNA structures and it is also intrinsic to DNA strand separation [73]. A long α-helical linker connects the wedge domain to the helicase domains. These C-terminal domains contain the helicase motifs, and couple the energy associated with ATP binding, hydrolysis, and product release, to enzyme motion, DNA unwinding and rewinding and fork clearing [64, 72, 74].

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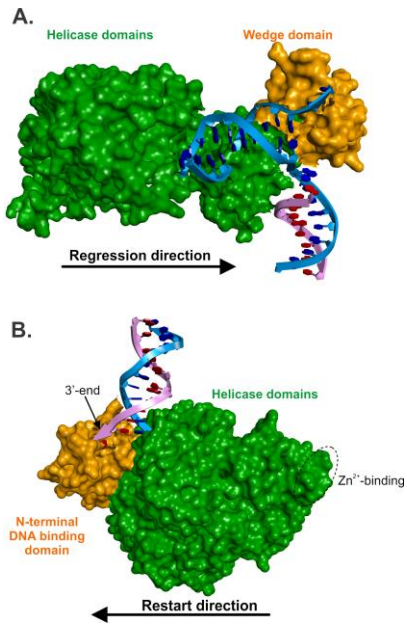

Figure 2. Replication fork rescue helicases . (A), RecG and (B), PriA. Each enzyme is represented as a Connolly surface. For RecG, a molecular model of the *E.coli* enzyme is shown with the wedge domain colored orange and helicase domains in green. Adapted from reference[58]. For PriA, the N-terminal DNA binding domain is colored orange and the helicase domains are colored in green. The location of the zinc binding sites are indicated by the dashed circle. Adapted from reference [82].

b. PriA – the restart specialist

Primosomal protein A (PriA) was originally known as factor Y and as protein n' and is required for the conversion of single-stranded ϕX174 DNA to the duplex, replicative form, in an *in vitro*-reconstituted system (reviewed in [75, 76]). Here PriA binds to an ssDNA hairpin structure in ϕX174 called n'-primosome assembly site (PAS), leading to the subsequent assembly of the primosome, a complex responsible for primer RNA synthesis and duplex DNA unwinding at a replication fork [76, 77]. As the DNA sequence of PAS is unique, PriA is either a structure- or sequence-specific DNA binding protein. The formation of the primosome occurs in an ordered fashion [76]. Following binding of PriA to PAS, the complex is then recognized and bound by PriB, PriC, and DnaT. Formation of the primosome proceeds with the subsequent actions of DnaB, DnaC, DnaT and primase [75, 76].

In addition to its role in ϕX174 DNA replication, genetic analyses were used to demonstrate that **PriA** plays a crucial role in DNA replication fork rescue [21, 75, 76, 78]. Null mutations in ***priA*** resulted in a complex phenotype that includes constitutive induction of the SOS response, defects in the repair of UV-damaged DNA, DNA double-strand break repair and homologous recombination, and these mutants exhibit defects in both constitutive and induced stable DNA replication [33, 79-82]. Collectively, the data supported a key role for PriA in replisome assembly at sites distinct from *oriC* in a process that facilitates replication restart following fork stalling and/or DNA breakage [75, 83, 84].

The 82kDa PriA DNA helicase consists of two domains (Fig. 2b and [85-87]). The N-terminal 181 aa are associated with DNA binding while the C-terminal 551 aa contains the ATP binding and DNA helicase motifs which are interrupted by two, C4-type zinc finger motifs. These Zn-finger motifs are essential for *in vitro* primosome assembly on PAS, for recombination-dependent DNA replication *in vivo* and, for interactions with other primosomal proteins [88-90].

The DNA binding properties of PriA, mediated by the N-terminus, are consistent with its activity at stalled replication forks. It binds with high affinity to D-loops and to model, fork structures *in vitro* [91-93]. This binding is mediated through specificity for DNA strands with accessible 3'-ends [94] [87]. PriA has been assigned to helicase Superfamily 2 and has been shown to unwind DNA with a 3'→ 5' polarity *in vitro* [95, 96]. DNA unwinding is fueled by the hydrolysis of ATP (dATP), is site-specific (*i.e.*, PAS), structure-specific, and ssDNA-dependent [97]. Also, DNA

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unwinding of model fork substrates is stimulated by SSB. This stimulation involves both a physical and functional interaction between the two proteins [51, 52]. Here, like that demonstrated for RecG, the OB-fold of PriA binds to the linker domain of SSB [68]. This interaction is essential to the loading of PriA by SSB onto model forks substrates, which also similar to RecG and results in remodeling of the helicase [3].

Once loaded onto a stalled replication fork PriA displays two types of activities. The helicase activity unwinds lagging-strand DNA present at the fork thereby generating a single-stranded DNA binding site for DnaB [92]. The second activity is the loading of DnaB onto the lagging-strand template *via* a complex series of protein-protein interactions reminiscent of primosome assembly for ϕX174 DNA [21, 75, 76]. Here it facilitates assembly of a multi-protein complex that includes PriB and DnaT. Intriguingly, while the helicase activity is required to generate ssDNA for DnaB, it is not required for the loading process itself [21, 98]. During loading, the replicative helicase, DnaB, is transferred from a DnaB-DnaC complex onto ssDNA that can be exposed or SSB-coated. Once DnaB has been loaded, a new replisome forms, leading to the resumption of DNA replication [13, 75].

c. SSB – the mediator of DNA transactions at forks

The single-stranded DNA binding protein (SSB) is essential to all aspects of DNA metabolism in *Escherichia coli* [99-103]. SSB has dual roles that are interconnected. First and as its name indicates, the protein binds to and stabilizes single-stranded DNA (ssDNA) intermediates generated during DNA processing. Second, it binds to as many as seventeen proteins temporally and spatially, to both store and target enzymes to the DNA when needed [51, 104]. These binding partners are collectively known as the "SSB-interactome" and include Exonuclease I, Alkylation protein B, the χ-subunit of DNA polymerase, DnaG, RecO, uracil glycosylase, topoisomerase III and the PriA, RecG and RecQ DNA helicases [104-106]. As many of the interactome partners including SSB contain OB-

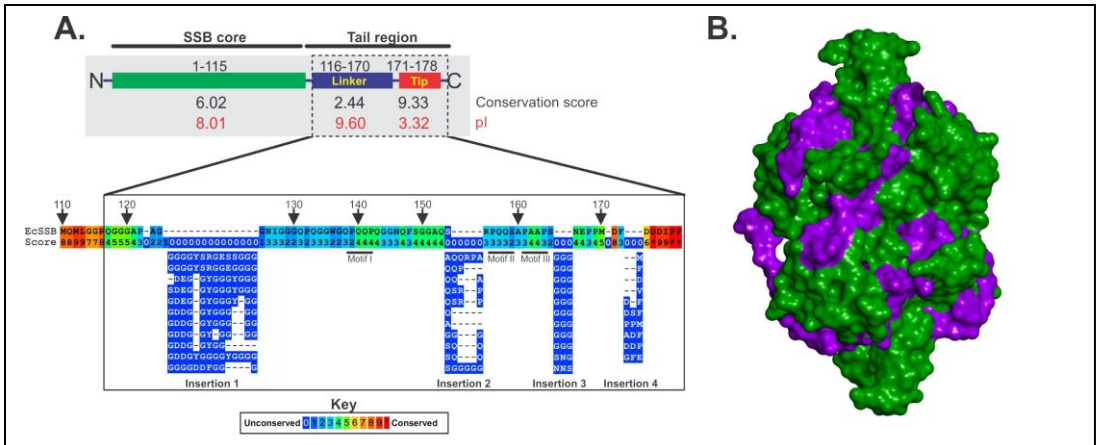

Figure 3. Organization of the SSB protein. (A), Schematic of SSB divided into the core and tail regions by proteolytic cleavage. The conservation scores for each region were calculated from alignments using Praline [130]. The pI of each region is shown in red and was calculated using the ProtParam tool of Expasy [131]. The pI of the intact protein is 5.44. (B), The SSB tetramer is intimately associated with ssDNA. The image was generated using PDB file 1EYG [104]. The DNA is colored purple and the tetramer, is colored green. Both protein and DNA are represented as Connolly surfaces.

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folds, the SSB interactome has been classified as the first OB-fold family of genome guardians in *E. coli* [68].

SSB exists as a homo-tetramer with a monomer MW of 18, 843 Da [107]. Each monomer can be divided into two domains defined by proteolytic cleavage: an N-terminal domain comprising the first 115 residues and a C-terminal domain spanning residues 116 to 178 (Figure 3A and [108]). The N-terminal domain is responsible for tetramer formation and binding to ssDNA which is mediated by the four OB-folds in the tetramer. Here, ssDNA binding by the OB-folds results in the wrapping of the polynucleotide around the SSB tetramer (Fig. 3B and [26, 109]). In addition, the OB-fold is also responsible for binding to the linker domain of nearby SSB tetramers [68]. Molecular modeling was used to show how the linker could bind to the OB-fold (Fig. 3b, the red ligand in the upper left tetramer). The role of inker/ OB-fold binding in SSB function is explained below.

The disordered, C-terminal domain can be further subdivided into two additional regions: a sequence of approximately 50 amino acids that has been called the intrinsically disordered linker (IDL) or linker which has a pI of 9.6 (Fig. 3A and [99, 103, 110, 111]). The overall sequence conservation of the linker is poor and this is due to the presence of one to four insertions of different lengths affecting the overall alignment (Fig. 3A and [111]). This is immediately followed by the acidic tip or, the last 8-10 residues which are very well conserved and is overall, acidic with a pI of 3.32 ("tip"; [99, 110]).

This acidic tip has long been thought to be the primary and only, protein-protein interaction domain of SSB (for review see [99]). Surprisingly, studies using intact proteins show that the acidic tip is not required for binding at all [68, 112-114]. Instead, it functions to regulate the structure of the C-terminal domain using long-range electrostatic effects [115]. These effects ensure that the linker does not associate with the tetramer from which it emanates, making it available for partner binding. Consequently, when the acidic tip is mutated or deleted, the linker collapses back onto the SSB OB-fold, thereby inactivating the protein [114]. Mutant linker/OB-fold binding is rescued by high affinity, ssDNA-binding which results in a conformational change in SSB that exposes the C-termini and makes available for partner binding [105, 116]. Consistent, the affinity of SSB for the chi subunit of DNA polymerase and separately for PriA, increased when SSB was pre-bound to ssDNA [105]. Further support of the collapsing of mutant C-termini back onto the SSB tetramer, came from a recent atomic force microscopy study which showed that the volume of wild type SSB is 3-fold higher than that of SSBΔC8, a mutant which lacks the acidic tip [3].

As the acidic tip is not required for partner binding, a separate region of SSB must be involved and this is the linker domain [68]. Initial analysis of the primary amino acid sequence of the C-terminal domain of SSB revealed several insights. First, the region is over-represented in glycine, glutamine and proline residues (Fig. 4A and [111, 114]). Second, several of these are arranged in repeats with as many as seven spider silk motifs. These are proposed to impart both flexibility and tensile strength to the linker, enabling SSB to bind to partners ranging in size from 20-80kDa and to itself to produce a stable complex on ssDNA (Fig. 4d). Third, the N-terminal half of the linker can be modeled on a collagen strand further supporting the idea that these elements contribute to the flexibility of the linker (Fig. 4B and [114]). Fourth, and perhaps most importantly, the analysis revealed the presence of three, conserved PXXP motifs (Fig. 4a, motifs highlighted by red boxes).

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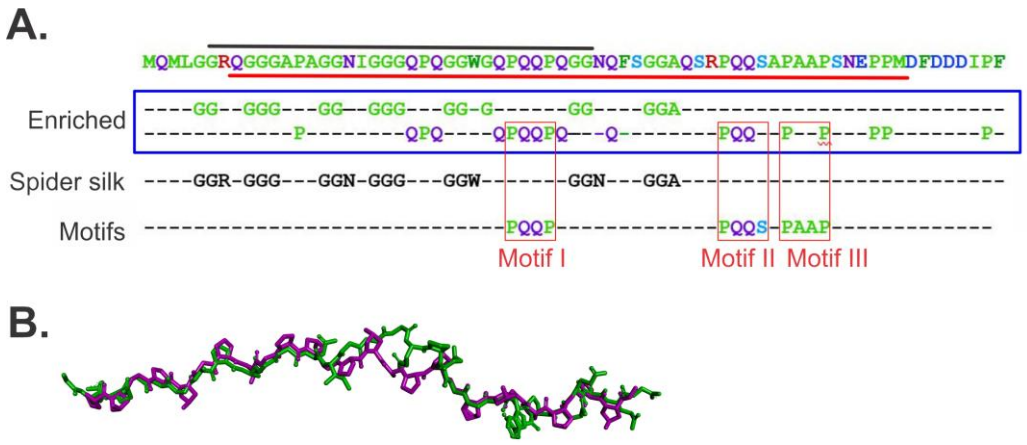

Figure 4. The linker domain of SSB contains sequence elements critical to its function. (A). The sequence of the C-terminal 69 residues of *E. coli* SSB is presented in the first line. The black line above the sequence corresponds to the putative polyproline type II helix which terminates at residues 148 and 149 as these are known to disrupt these helices [132]. The red line demarcates the residues that form the linker domain. Enriched, the most over-represented residues highlighted in lines a and b. Residues 148 and 149 are highlighted to indicate where the PPII-helix would. Sequence analysis of the protein was done using REPRO at http://www.ibi.vu.nl/programs/ to determine the presence of repetitive elements. The spider silk sequence motifs which are repeated seven times, are indicated. The location of the three PXXP motifs is highlighted by the red boxes. (B). Homology modeling reveals that residues 116-145 can adopt a collagen-like structure. The model of the N-terminal part of the SSB linker (green) is shown superimposed onto a collagen strand (purple).

In eukaryotic cells, PXXP-containing ligands bind Src homology 3 (SH3) domains to facilitate intracellular signaling [117-120]. Critically, SH3 domains are structurally almost identical to OB-folds with the folds aligning very well with an average root mean square deviation of less than 2.0 Å for the β-strands [121]. Importantly, OB-folds are present in both SSB and as many as twelve interactome partners [49, 64, 114, 122-125]. Consequently, binding involves the docking of the linker of SSB into the OB-fold present in the partner protein (Fig. 5 and [68, 114]). When the interactions take place between the PXXP motifs in the linker of one SSB tetramer and one or more of the OB-folds in nearby SSB tetramers, cooperative ssDNA binding occurs (Fig. 5, top panel). When these interactions occur between the linker-PXXP motifs of an SSB tetramer and the OB-fold in an interactome partner such as RecG, loading of that protein onto DNA takes place. It is not surprising then that mutations in key residues of the RecG OB-fold or in the PXXP motifs of SSB, eliminate helicase binding and cooperative ssDNA binding, respectively [114].

This mechanism of binding has been termed the linker/OB-fold interaction model [114]. Support for this model comes from several research groups who have demonstrated that the linker imparts species-specific, partner binding [112, 114, 126]. Finally, the binding site of Alkylation protein B mapped to PXXP motifs II and III of SSB [113]. An important caveat of this model is that linker and DNA binding to either SSB or partners is competitive and this has important implications for interactome function as explained below.

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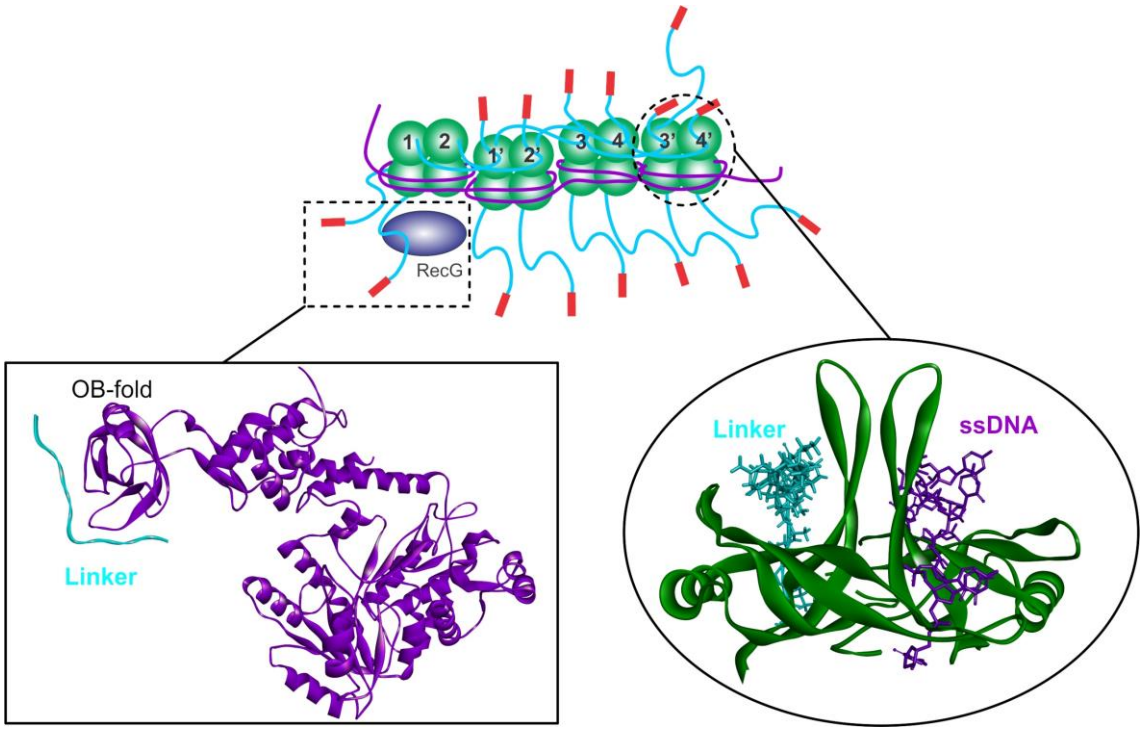

Figure 5. The linker/OB-fold network of interactions is critical to SSB interactome function. Top, a schematic of an SSB-ssDNA complex demonstrating how the linker-OB-fold network of interactions produces a stable complex where the ssDNA is coated and protected. Here, the linker from the first tetramers bind to the OB-folds in adjacent tetramers, while other C-termini are exposed and available for partner binding, in this case RecG (left inset). Here, the helicase is presented as a ribbon diagram (purple) and a linker domain (cyan) is modeled into the OB-fold. When SSB-tetramers interact, some OB-folds are occupied by linker domains (cyan) while others bind ssDNA (purple). For clarity, only two monomers of the tetramer are shown and represented as ribbon diagrams (colored green). The schematic is adapted from [110].

4. SSB-DNA helicase interactions during loading

a. SSB-RecG

It is known that SSB and RecG bind to one another both *in vivo* and *in vitro* [48, 51]. Mechanistic insight into the dynamics of this interaction has been provided by atomic force microscopy (AFM) studies [24]. The preferred substrate for RecG, which is a fork with a gap in the leading strand, was used to visualize loading by SSB (Figure 6A and [47, 48, 127, 128]). In these studies, SSB was bound to the fork first, followed by the helicase. RecG and SSB appear with different contrasts on AFM images, with SSB appearing larger than RecG by a factor of 2 [24]. Thus, SSB appears as a large blob and RecG as the smaller blob (Fig 6b-e; blue and green arrows, respectively). Further, SSB is always bound at the fork with RecG positioned on average, 36bp upstream of the fork (Fig. 6f). This positioning on the parental duplex region of the fork is the result of thermal sliding and is independent of a nucleoside triphosphate. Further evidence of sliding came from a study using high-speed AFM, where loading of RecG by SSB was followed by the helicase sliding on the parental duplex DNA [72].

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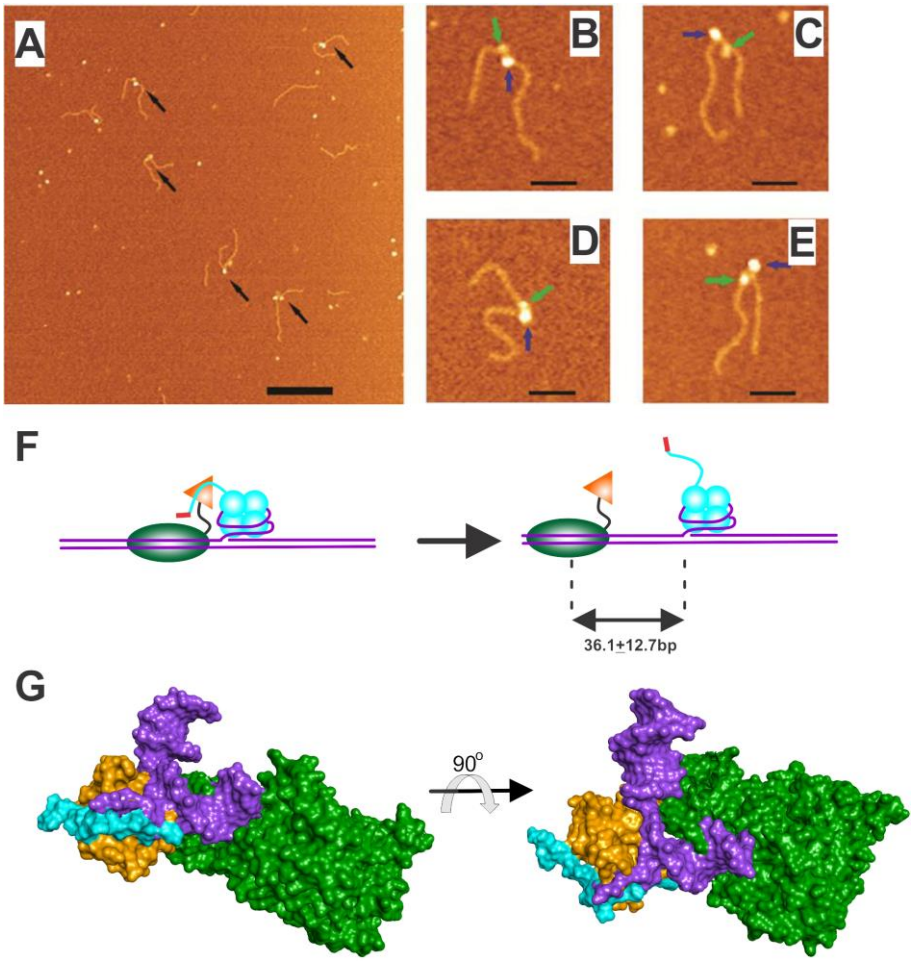

Figure 6. SSB loads RecG and remodels the helicase in the process. (A) Large scale atomic force microscopy image of SSB-RecG-DNA complexes. Proteins are indicated by arrows. Scale bar is 200 nm. B-E, Zoomed images of four double-blob complexes. Large (SSB) and small (RecG) blobs are indicated with blue and green arrows (scale bars 50 nm). (F). A model for RecG loading by SSB. SSB binds to the fork first and to RecG via the linker from one monomer within the tetramer. Loading ensues, concomitant with remodeling of the wedge domain (orange) so that only the helicase domains (green) can bind DNA. Once loaded, RecG slides, using thermal energy, on average, 36bp ahead of the fork. (G). Molecular models of RecG bound to a fork (purple) and the SSB linker (cyan; a PXXP-containing ligand). The wedge domain is colored orange and helicase domains in green to match the schematic in panel F. As fork and linker binding are competitive, the cyan and purple colors overlap demonstrating that that cannot occupy the same space. The two images are rotated 90° relative to each other to enable clear visualization. AFM images are from [40].

During loading, the linker of fork-bound SSB binds to the RecG OB-fold (Fig. 6g). As the wedge domain of the helicase is essential for fork binding, the only way that dsDNA binding followed by thermal sliding can occur is if the helicase domains are the only parts of the RecG associated with the DNA [73]. Therefore, when the linker of SSB binds to the RecG OB-fold, remodeling of the helicase occurs thereby enabling it to bind to and slide on, the parental duplex DNA. This sliding plays an important role in fork rescue. If the parental duplex DNA near the fork is damaged or otherwise modified, RecG binding is impaired [1]. This may serve as a signal for RecG to disengage from the

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DNA, and for the repair machinery to repair the damage, before the onset of fork regression and/or replication restart.

b. SSB-PriA

A similar AFM approach was used to visualize PriA fork interactions and the effects of SSB on helicase loading. As ATP influences the overall structure of PriA in AFM, studies were done in the absence of a nucleoside cofactor [3]. As for RecG, in the absence of SSB, PriA binds exclusively to forks but prefers a fork with a gap in the nascent leading strand. This result is in contrast to previous work which demonstrated a preference for a fork DNA substrate with a gap in the nascent lagging strand [129-132]. This suggests that ATP may play an important role in dictating the specificity for PriA for fork substrates. This effect was not observed for RecG [24].

Visualization of SSB loading of PriA revealed intriguing insight (Figure 7). First, SSB is always observed bound to the fork and appears as a large blob (blue arrows in insets Ai and ii; and Bi and ii). Second, and as a consequence of remodeling, PriA is observed as a small blob in distinct positions relative to SSB (green arrows in the insets). Furthermore, when the DNA substrate has a gap in the leading strand, PriA is loaded onto all fork arms with equal probability. In contrast, when the substrate has a gap in the lagging strand, remodeled PriA is loaded preferentially to the leading strand. In contrast, in the absence of SSB, PriA binds exclusively to forks with ssDNA gaps. These results suggest that like RecG, PriA is remodeled by SSB so that it can now bind to duplex DNA. This may facilitate, the binding and/or translocation of PriA along the duplex may stimulate the association of PriA at the stalled replication fork in an ATP-independent way, facilitating the restart process once the ATP is available for PriA helicase activity.

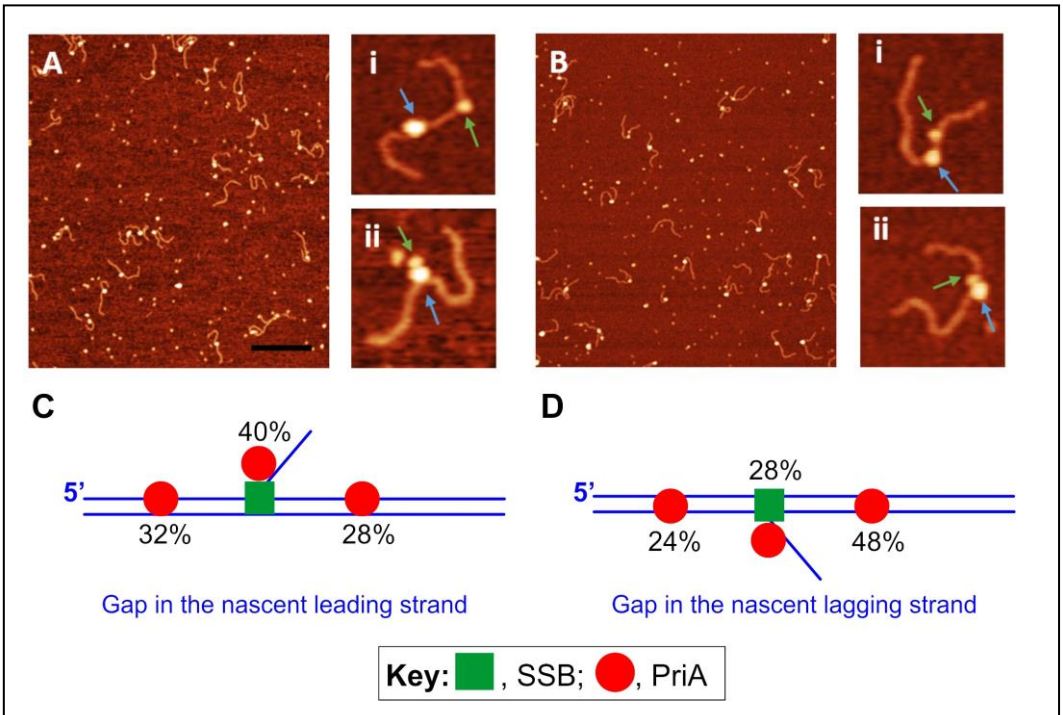

Figure 7. SSB loads PriA and remodels the helicase in the process. A and B, AFM images of PriA loading by SSB. The large blobs correspond to SSB (blue arrows) while the smaller ones correspond to PriA (green arrows). The insets in panels A and B are zoomed images. C and D, PriA locations following SSB loading and remodeling. The values represent the frequency of occurrence of PriA in each location. Adapted from reference [46].

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5. The mechanics of fork regression by RecG

Once loaded at a stalled replication fork, RecG is thought to regress these away from the site(s) of DNA damage. Stalled replication fork regression is a process that involves the net backward movement of the fork, away from the site of DNA damage, concomitant with the unwinding of nascent heteroduplex arms to form a 4-stranded, Holliday Junction-like structure or "chicken foot" (Figure 1a). This requires a specialized DNA helicase that must have the ability to couple unwinding of the nascent leading and lagging strand arms to duplex rewinding (Fig. 1B). Two regions can be simultaneously rewound, provided complementary strands are available. These are the parental duplex and a fourth arm, known as the middle toe of the chicken foot that is formed between the leading and lagging strand arms. Also, this specialized DNA helicase must be able to generate sufficient force to clear the fork of potentially numerous protein obstacles such as SSB (Fig,1C).

Single-molecule studies using magnetic tweezers revealed that this enzyme is the RecG DNA helicase [1]. Using a 1,200bp hairpin, RecG was shown to rewind complementary fork arms; to couple DNA unwinding to this rewinding and to generate Holliday junctions in the process (Figure 8). Importantly, RecG catalyzed fork regression only. During the coupled unwinding/rewinding reaction, that is fork regression, movement of the fork proceeded at a rate of 269± 2 bp/sec and with a processivity of 480 ± 20 bp. As RecG utilizes 1 ATP to translocate 3bp, 160 nucleoside triphosphate molecules are hydrolyzed on average per fork regression event [42, 133]. By using a combination of both magnetic and optical tweezers, the authors demonstrated that RecG can catalyze rewinding against forces of up to 35pN, with only a moderate drop of about 40% in rate (Fig. 8C). This is consistent with RecG being a very powerful motor that should be able to clear the fork of bound proteins. This was tested using SSB, which requires at a minimum, 10pN of force per tetramer. Not surprisingly, the protein posed little threat to RecG as the enzyme was capable of coupling rewinding to efficient SSB displacement (Fig. 8D). Subsequent studies determined that the SSB linker is required for efficient displacement as mutant SSBs with the linker either partially or completely deleted, significantly impaired the ability of RecG to displace them (Fig. 8E). This does not require linker/OB-fold interactions as the RecG Ob-fold is already bound to the fork. Instead, this reflects the ability of RecG to push SSB off the DNA. When linker/OB-fold, SSB-SSB interactions are in play as they are for wild type and SSBΔC8, the pushing by RecG is communicated between the SSB tetramers SSB-ssDNA facilitating displacement. In contrast, when SSB-SSB linker/OB-fold interactions are absent, the SSB_{125} tetramers which have no linker function as separate, tightly bound entities that impede RecG translocation.

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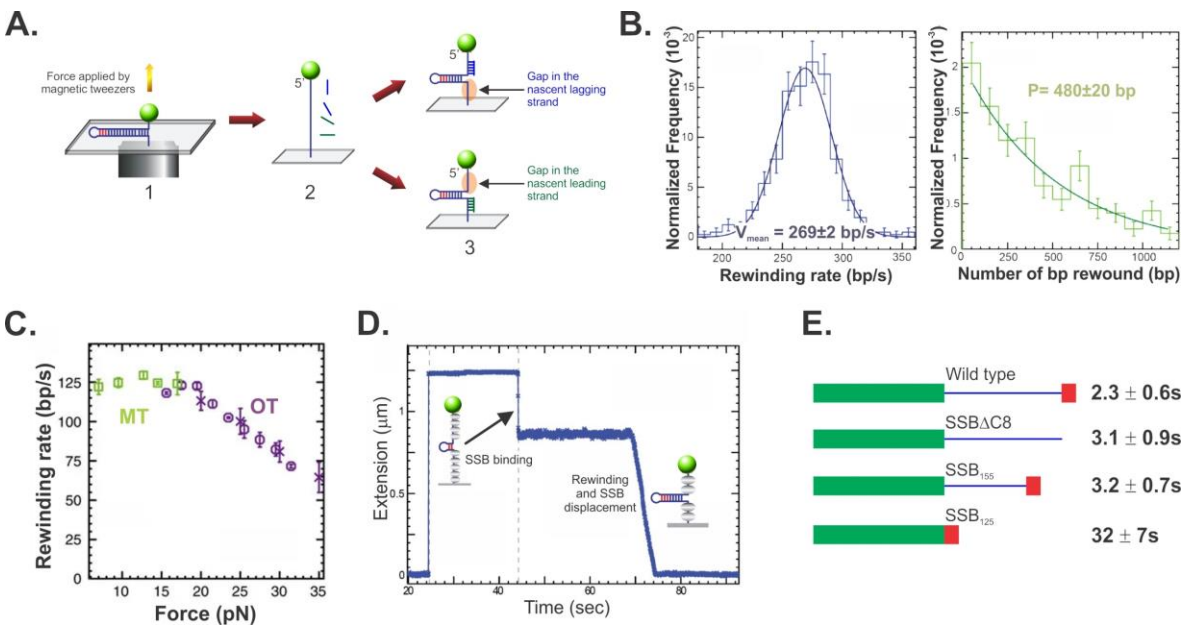

Figure 8. RecG catalyzes the key steps required for fork regression and displaces SSB in the process. (A) Construction of forks with gaps in either the leading or lagging strands. Here, the 1,200 bp hairpin is fully stretched by the application of force from the magnetic tweezers. Then, oligonucleotides complementary to the 5′- or 3′-proximal regions are introduced in separate reactions and allowed to bind. Once the opposing force is decreased, a partial hairpin is extruded and as the oligonucleotide remains annealed to reveal a fork with a gap on the opposite side, either the lagging (top) or leading strand (bottom). B, Rates and processivity of RecG-catalyzed fork rewinding at 37°C. P, processivity. C, RecG can work against large opposing forces during fork rewinding. MT, data from magnetic tweezer experiments. OT, data from assays using optical tweezers; D, RecG readily displaces SSB during fork rewinding. Here, the hairpin is fully unwound by the application of force. When SSB is added, binding results in wrapping of the polynucleotide around the tetramers. This causes shortening of the DNA tether. E, Functional linkers are required for SSB displacement by RecG during fork rewinding. Each SSB is colored green (N-terminal domain), blue (linker) and red, acidic tip. SSB_{155} and SSB_{125} are mutant proteins where the linker was either partially or completely removed, respectively. The values next to each protein, represent the lag time for rewinding to initiate following the addition of RecG. Data panels B-D are from [42] and panel E is from [133].

6. Summary

Stalled DNA replication fork rescue is an essential process. It requires an intimate association between the SSB protein and the DNA helicases, PriA and RecG. This association is critical to the loading of these enzymes onto stalled forks, enabling them to out-compete other proteins. During the loading process, each helicase is remodeled by SSB. For RecG, the wedge domain is bound to the linker domain of SSB and cannot gain access to the fork. Consequently, the helicase domains of RecG mediate binding to the DNA duplex followed by thermal sliding ahead of the fork, possibly clearing bound proteins in its immediate vicinity. Once RecG is loaded, it catalyzes an efficient regression reaction in the presence of ATP. Here, the enzyme works against large opposing forces, coupling fork rewinding to the displacement of tightly bound proteins such as SSB. RecG is unique in its fork function, catalyzing fork regression only producing Holliday junctions for subsequent

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processing by additional enzymes such as RuvAB. Once this additional processing has taken place and the original fork structure restores, PriA is loaded onto the DNA, resulting in the ultimate reloading of the replisome. For PriA, the N-terminal DNA binding domain is also bound by the linker domain of SSB during loading. Consequently, the helicase is loaded onto the fork arms with the final location dictated by the presence of single-stranded DNA in the opposite arm. Similar to RecG, this binding is mediated by the helicase domains of PriA. Collectively, the interactions between SSB and these two critical helicases results in restoration of the fork and the restart of DNA replication.

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