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Site-Directed Mutagenesis and Yeast Reverse 2-Hybrid-Guided Selections to Investigate the Mechanism of Replication Termination

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1. Introduction

DNA replication in prokaryotes, in budding yeast and in mammalian DNA viruses initiates from fixed origins (*ori*) and the replication forks are extended in either a bidirectional mode or in some cases unidirectionally (Cvetic and Walter, 2005; Sernova and Gelfand, 2008; Wang and Sugden, 2005; Weinreich et al., 2004). In higher eukaryotes there are preferred sequences located in AT-rich islands that serve as origins (Bell and Dutta, 2002). In many prokaryotes, the two replication forks initiated at *ori* on a circular chromosome meet each other at specific sequences called replication termini or *Ter* (Bastia and Mohanty, 1996; Kaplan and Bastia, 2009). The Ter sites bind to sequence-specific DNA binding proteins called replication terminator proteins that allow forks approaching from one direction to be impeded at the terminus, whereas forks coming from the opposite direction pass through the site unimpeded (Bastia and Mohanty, 1996, 2006; Kaplan and Bastia, 2009). Therefore, the mode of fork arrest is polar. The polarity of fork arrest in *Escherichia coli* and *Bacillus subtilis* is caused by the complexes of the terminator proteins called Tus and RTP (Replication Terminator Protein), respectively, with the cognate *Ter* sites to arrest the replicative helicase (such as DnaB in case of *E. coli*) in a polar mode (Kaul et al., 1994; Khatri et al., 1989; Lee et al., 1989; Sahoo et al., 1995). What is the mechanism of polar fork arrest and what might be the physiological functions of *Ter* sites? Using *E. coli* as the main example, with the aid of the techniques of site-directed mutagenesis, yeast reverse 2-hybrid based selection of random mutations (described below), and biochemical characterizations of the mutant forms of the Tus protein, many aspects of the mechanism of replication fork arrest at Tus-*Ter* complexes have been determined. This and a brief description of the current state of the knowledge of replication termination in eukaryotes have also been reviewed below.

Replication termini of *E. coli* **and the plasmid R6K:** Sequence-specific replication termini were first discovered in the drug resistance plasmid R6K (Crosa et al., 1976; Kolter and Helinski, 1978) and in its host *E. coli* (Kuempel et al., 1977). The terminus region of R6K was identified and sequenced (Bastia et al., 1981) and subsequently shown to consist of a pair of *Ter* sites with opposite polarity (Hidaka et al., 1988). An *in vitro* replication system was

developed in which host cell extracts initiated replication of a plasmid DNA template and the moving forks were arrested at the *Ter* sites (Germino and Bastia, 1981). It was also suggested that a terminator protein that might cause fork arrest was likely to be host-encoded. Subsequently, the open reading frame (ORF) encoding the terminator protein was cloned and sequenced and the gene was named TUS (Terminus Utilizing Substance) (Hill et al., 1989). Tus protein was purified from cell extract of *E. coli* and shown to bind to the plasmid *Ter* sequences (Sista et al., 1991; Sista et al., 1989). The *TerC* region of *E. coli* was found to contain several *Ter* sites in two sets of 5 sites each with one cluster having the opposite polarity of fork arrest in comparison with that of the second set (Hill, 1992; Pelletier et al., 1988). Together, these sequences formed a replication trap (Fig.1A). For example, if the clockwise moving fork got arrested at *TerC*, it waited there for the counterclockwise fork to meet it at the site of arrest. The *Ter* consensus sequence is shown in Fig.1B. Site-directed mutagenesis showed the bases that are critical for Tus binding (Duggan et al., 1995; Sista et al., 1991). The complete process of initiation, elongation and termination has been carried out *in vitro* with 22 purified proteins that were necessary and sufficient for fork initiation, propagation and termination (Abhyankar et al., 2003).
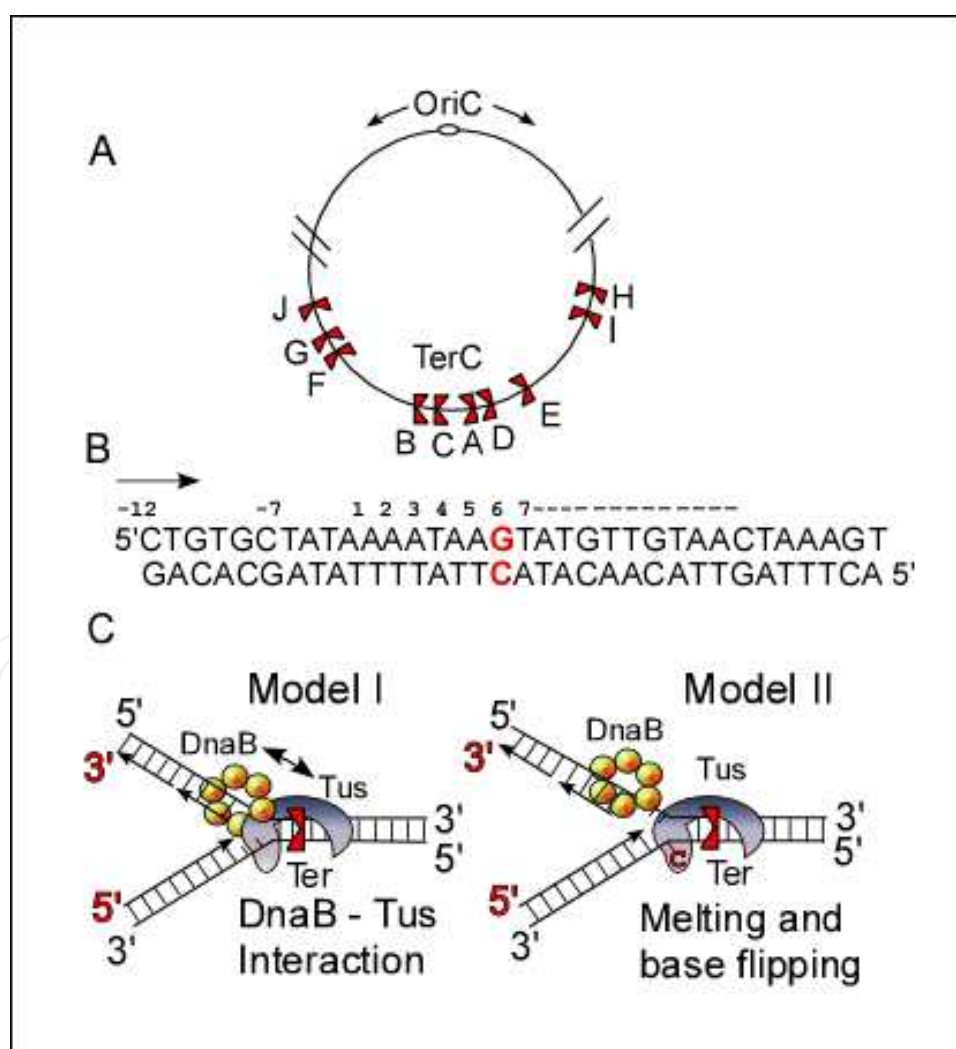

Fig. 1. Replication termini of *E. coli*. A, The bacterial replicon showing the origin and the *TerC* region at its antipode. The flat surfaces of the *Ter* sites indicate the permissive face and

the notched one the nonpermissive face; B, consensus Ter sequence showing the blocking end at the left (arrow) and the nonblocking end at the right; the red C on the bottom strand was reported to flip out upon Tus binding; C, two models of polar fork arrest. Model 1 postulates that both Tus binding to *Ter* and interaction or contact between the nonpermissive face of the Tus-*Ter* complex with DnaB helicase causes polar arrest; model 2 suggests that it is strictly the Tus-*Ter* interaction and the partial melting of the DNA catalyzed by DnaB and the flipping of C6 that causes strong affinity of Tus for *Ter*. The helicase approaching the permissive face fails to induce high-affinity binding of Tus to *Ter*.

Using an *in vitro* helicase assay catalyzed by purified DnaB and Tus proteins, it was shown that Tus binding to *Ter* acts as a polar contra- or anti-helicase and arrests helicase catalyzed DNA unwinding in one orientation of the Tus-*Ter* complex while allowing the helicase to pass through mostly unimpeded in the opposite orientation (Khatri et al., 1989; Lee et al., 1989). It was also shown that the RTP of *B. subtilis* arrested *E. coli* DnaB helicase at the cognate *Ter* sites of the Gram-positive bacterium *in vitro* was able to arrest DnaB of *E. coli* in a polar mode. However, it did not arrest rolling circle replication of a plasmid (Kaul et al., 1994). It is of some interest that not all helicases were arrested at Tus-*Ter* complexes because helicases such as Rep and UvrD were not arrested by either orientations of Tus-*Ter* (Sahoo et al., 1995). The Tus-*Ter* complex of *E. coli* could arrest forks with a very low efficiency *in vivo* in the *B. subtils* host, as contrasted with their ability to arrest forks more efficiently in the natural host. In addition to DnaB, RNA polymerase of bacteriophage T7 and *E. coli* were also arrested in a polar mode, by the Tus-*Ter* complex (Mohanty et al., 1996, 1998). This latter observation had raised the possibility that the Tus-*Ter* complex might just be a steric barrier to unwinding because enzymes apparently as diverse as DnaB helicase and RNA polymerases were arrested by the same complex. This mechanistic issue has been discussed in more detail later.

Crystal structures of Terminator proteins: The first crystal structure of a terminator apoprotein, namely that of RTP, showed that the protein was a symmetrical winged helix dimer (Fig.2B) (Bussiere et al., 1995). The *Ter* sites of *B. subtilis* contain overlapping core and auxiliary sequences with each site binding an RTP dimer (Hastings et al., 2005; Smith and Wake, 1992; Wilce et al., 2001). How can a symmetrical protein arrest forks with polarity? This question was subsequently answered when the crystal structure of two dimeric RTPs bound to a complete bipartite *Ter* site was solved (Wilce et al., 2001). It was shown that the structure of the protein-DNA complex is different at the core complex as contrasted with that of the adjacent auxiliary complex. The crystal structure of Tus bound to *Ter* DNA showed a bi-lobed protein with a positively charged cleft formed by several beta strands that contacted the major groove of the DNA and distorted the latter from the canonical structure (Fig.2A) (Kamada et al., 1996). The transverse view of Tus bound to a space-filling model of DNA shows that the face that arrests replication forks and DnaB has a loop called the L1 loop. The L1 loop appears to play a critical role in fork arrest.

Tus-DnaB interaction: We performed yeast 2-hybrid analysis (described below), confirmed by *in vitro* affinity binding to immobilized Tus, to show that DnaB interacted with Tus (Mulugu et al., 2001). The principles of forward 2-hybrid (Fields and Song, 1989) and reverse 2-hybrid analysis (Mulugu et al., 2001; Sharma et al., 2001) are shown in Fig.3. The open reading frame (ORF) of a protein X is cloned in the correct reading frame to the transcriptional activation domain of Gal4 of yeast (pGAD424-X). A suspected interacting
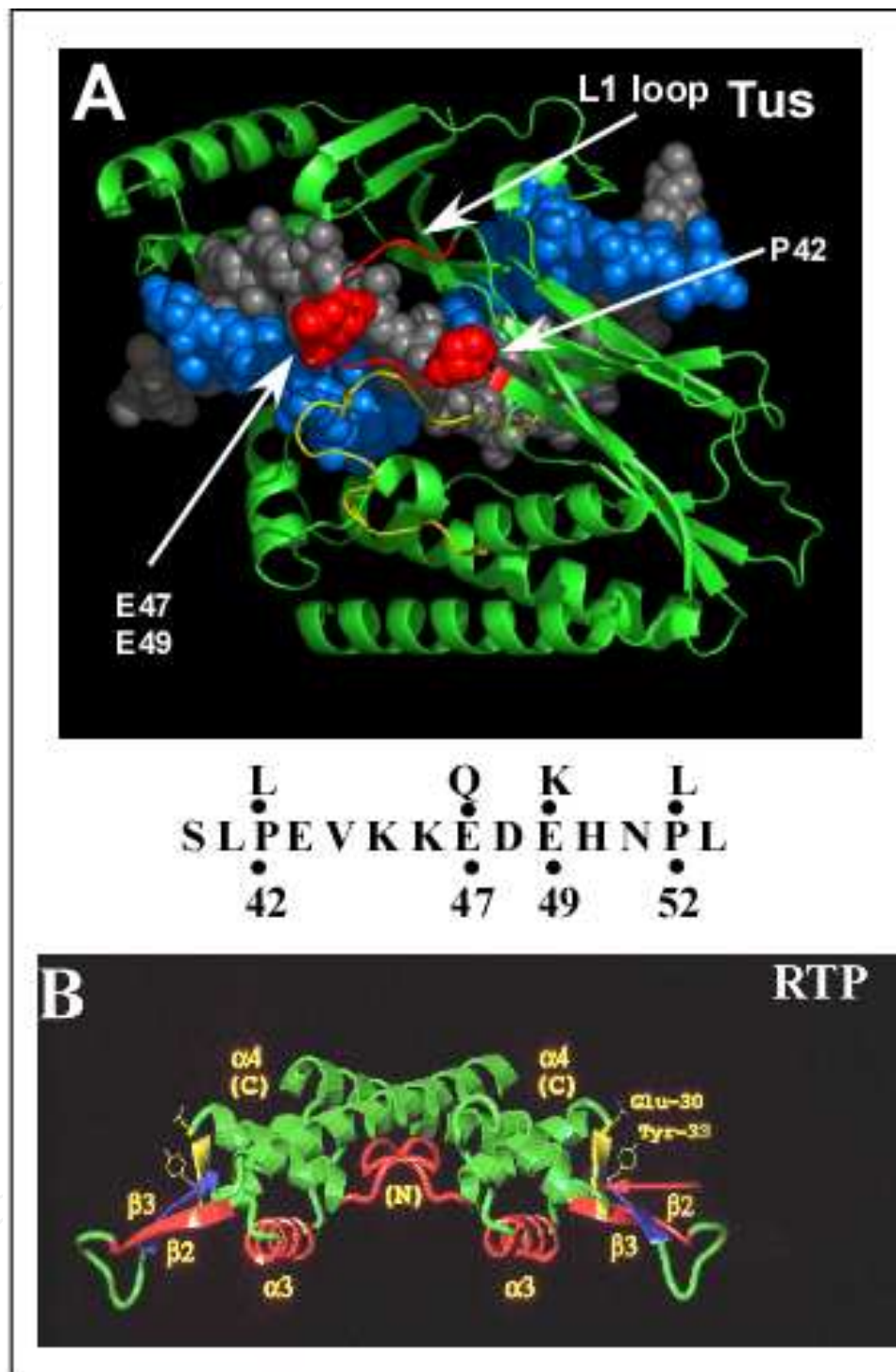
Fig. 2. Crystal structure of Tus-*Ter* complex of *E. coli* and RTP apoprotein of *B. subtilis*. A, crystal structure of Tus-*Ter* complex showing the blocking face with the L1 loop shown in red. Three residues, namely P42, E47 and E49, when mutated (see lower sequence) show impaired helicase arrest. P42L shows slightly reduced DNA binding; E47Q shows stronger DNA binding; and E49K shows no reduction in *Ter* binding but significant reduction in fork and helicase arrest. B, crystal structure of the RTP dimer apoprotein. The Tyr-33 arrow depicts a residue needed for the interaction of Tus with DnaB, as shown by a bifunctional labeled crosslinker that upon cleavage at an S-S bond transfers the label from RTP to DnaB.
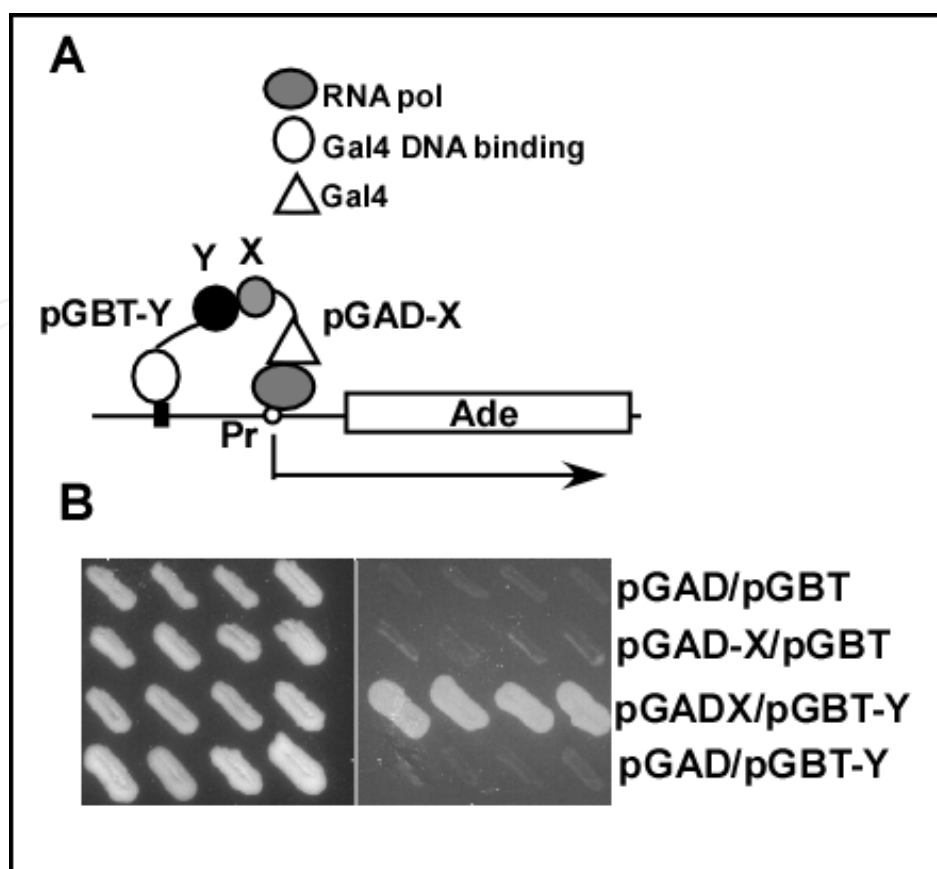
Fig. 3. Schematic representation of forward and reverse 2-hybrid selection. A, The plasmids pGBT-Y and pGAD-X interact through interacting proteins X and Y and turn on the *Ade* reporter gene leading to growth on adenine (ade) dropout minimal medium. Either X or Y is mutagenized by low-fidelity PCR and introduced by transformation in the presence of the other plasmid into the indicator yeast strain. B, X-Y interaction leads to growth on ade-minus plates, and mutants that fail to interact show lack of growth on the selective plates. Trivial mutations, *i.e.*, those containing deletions, nonsense mutations, or frame-shifts are eliminated by Western blotting of cell extracts expressing the presumed X or Y mutant form. Candidates are further characterized by functional and biochemical analyses.

protein Y is similarly fused in-frame to the ORF of the DNA binding domain of Gal4. The yeast strain contains a transcriptional reporter (*Ade*) that is placed next to a promoter and the binding site for the Gal4 DNA binding site. Neither pGAD424-X nor pGBT9-Y can activate the transcription of the reporter gene. However, when both plasmids, each containing a different marker (*e.g.*, *Leu* and *Trp*), are transformed into the reporter yeast strain, X-Y interaction activates the reporter gene. Both plasmids are shuttle vectors that contain an *ori* active in *E. coli* and also an *ori* (*ars*) of yeast. The transcription and translation of the adenine (*Ade*) reporter causes the yeast cells to grow in an adenine dropout minimal medium plate. The reverse 2-hybrid procedure was used to select for missense mutations that break X-Y interaction as follows. Low fidelity PCR amplification of X (or Y) introduces random mutations into the ORF. Then, for example, the mutagenized ORF of X in the pGAD424 vector is used to transform the *Ade* reporter yeast strain containing a resident pGBT9-Y plasmid. Colonies that have mutations that break X-Y interaction are initially selected as clones growing on *Leu⁻Trp⁻* medium but failing to grow on *Leu⁻Trp⁻Ade⁻* dropout

plates. The mutations are expected to be a mixture of unwanted ones (*e.g.* missense, nonsense, frame-shifts) and useful ones (missense). The potential mutant clones are grown, cell-free lysates made and subjected to Western blots after polyacrylamide gel electrophoresis and developed with primary antibody raised against X followed by secondary reporter antibody. All clones that fail to produce the protein of the expected length are discarded, and those producing full length X-GAD are saved for further analysis.

Usually, the mutants are confirmed by co-immunoprecipitation of cell lysates with the anti-Y antibody (Ab) retained on agarose beads, stripping of the wild type (WT) X (or mutant X that should be in the wash), separation by gel electrophoresis and visualization with anti-Y Ab. Naturally, the authentic non-interaction mutant forms of X should no longer bind to Y or bind poorly. These "pull down" assays are used to confirm the reverse 2-hybrid results. If the interaction of X and Y is necessary for a biological function (*e.g.*, fork arrest at Tus-*Ter* complex), the X mutants that do not interact with protein Y are then tested by 2-dimensional agarose gel electrophoresis (Brewer and Fangman, 1987, 1988; Mohanty et al., 2006; Mohanty and Bastia, 2004) to determine whether they show the expected biochemical property (in this case, failure to arrest replication forks) (Mulugu et al., 2001). The reverse 2-hybrid approach is a powerful method that can yield mutants that specifically disrupt protein-protein interaction between a pair of known interacting proteins. This procedure can be followed up by isolation of additional mutations isolated by site-directed mutagenesis of residues close to the protein domain (as determined by X-ray crystallography) that contained the mutations recovered from the reverse 2-hybrid approach. A specific example is given below. By mutagenizing Tus by PCR, we were able to collect a pool of random mutants. We performed reverse 2-hybrid analysis of the mutant pool and recovered the mutation P42L (proline at position 42 to leucine) that fails to interact with DnaB. However, a P42L mutation also affected Tus-*Ter* binding to some extent. We mutagenized other residues by site-directed mutagenesis to isolate E47Q (glutamic acid at position 47 to glutamine) and E49K (glutamic acid at position 49 to lysine) (Fig. 2 and 3). Both of the latter mutants were defective in interaction with DnaB and in fork arrest *in vitro*. Whereas the E49K mutant form bound to *Ter* with the same affinity as WT Tus, E47Q had a higher DNA-binding affinity but was defective in fork arrest *in vivo* (Mulugu et al., 2001).

The yeast forward and reverse 2-hybrid analyses followed by biochemical analysis of Tus, showed that it contacted DnaB probably at the L1 loop because the only mutations that impaired helicase arrest and fork arrest without abolishing or significantly reducing Tus-*Ter* interaction were found only at the L1 loop. Another line of evidence for specific replisome-*Ter* interaction is inferred from the observation that that Tus-*Ter* complex works with very low efficiency when placed in *B. subtilis* cells as contrasted with their fork arrest efficiency in *E. coli in vivo* (Andersen et al., 2000).

If there is protein-protein interaction between Tus and DnaB and if this is necessary for fork arrest, how does Tus also promote polar arrest of RNA polymerase, an enzyme apparently different in structure from DnaB? One possible explanation is that Tus might make an equivalent contact with RNA polymerase to inhibit its progression, or else a different mechanism could be operating here. It should, however, be clearly stated that this line of reasoning does not necessarily disprove the first explanation. Based on the data discussed above, we have suggested a model of fork arrest that involves not only stable Tus-*Ter* interaction, but also protein-protein contacts between the DnaB helicase and the L1 loop of Tus (Fig.1C and Fig.2).

Base flipping and DNA melting: An alternative explanation of polar arrest is suggested in model 2 (Fig.1C). X-ray crystallography of Tus bound to linear DNA had shown all Watson-Crick base pairing (Kamada et al., 1996). However, it was reported that a forked DNA that had single stranded regions when co-crystallized with Tus showed a flipped base (C6 in Fig 1C, model 2). It was suggested that both DNA melting and base flipping and the capture of the flipped base by Tus greatly enhanced Tus binding for *Ter* when the helicase approached the blocking end of the Tus-*Ter* complex. The enzyme, when approaching the complex from the non-blocking end, displaced Tus from *Ter*. This interpretation was based on binding studies of Tus to *Ter* on partially single-stranded DNA having a flipped C (Mulcair et al., 2006). Unfortunately, these binding studies were performed between 150 mM-250 mM KCl at which DNA replication and DnaB activity *in vitro* is inhibited by >90% . Curiously, when binding was performed closer to a physiological salt concentration that is permissive of DNA replication, this high binding affinity was greatly reduced to that of the interaction between linear double stranded Ter DNA and Tus (Kaplan and Bastia, 2009). It was therefore necessary to carefully test model 2 to determine its authenticity.

An Independent test of the melting-flipping model shows that it is unnecessary for polar fork arrest: We wished to rigorously test model 2, which postulated that DNA melting and base flipping together could explain polar fork arrest under a physiological salt concentration that permitted DNA replication to occur (Bastia et al., 2008). We reasoned that the model could be tested if one could temporally and spatially separate DNA unwinding by DnaB helicase from its ATP-dependent locomotion on DNA (double- or single-stranded). It is known that when encountering a linear DNA with a 5′ tail and 3′ blunt end, DnaB enters DNA with both strands passing through the central channel of DnaB (Kaplan, 2000). The translocation of DnaB on double-stranded DNA (dsDNA) requires ATP hydrolysis. We constructed the DNA substrate shown in Fig. 4. The DnaB helicase enters the substrate from the left by riding the 5′-single-stranded tail, slides over dsDNA containing a *Ter* site present in both orientations and upon reaching the forked structure with a 3′ overhang, DnaB unwinds this labeled strand (shown in blue). In the blocking orientation of Tus-*Ter* complex, the DnaB helicase slides on the dsDNA until it reached the *Ter* site, at which it is arrested, as shown by its failure to melt off the labeled 3′ tail shown in blue. In the reverse orientation of Tus-*Ter*, the DnaB sliding should displace Tus from *Ter* and continue sliding until it reached the 3′ overhang fork-like structure. At this point it should melt the labeled oligonucleotide, causing its release that can be resolved in a polyacrylamide gel at neutral pH and quantified (Fig.4). Our experiments showed that DnaB sliding, that involved no melting of DNA, not even a transient one, was arrested in a polar mode at a Tus-*Ter* complex. We proceeded to confirm the results further by introducing a pair of site-directed A-T inter-strand cross-links at two residues preceding C6. This covalent interstrand linkage prevented any chance of even transient DNA melting catalyzed by DnaB preceding the C6 residue. We confirmed that in such a substrate, DnaB sliding was arrested in a polar mode by the Tus-*Ter* complex only when present in the blocking orientation. These experiments led us to conclude that under physiological conditions a melting-flipping mechanism is not necessary (and probably does not occur) to cause polar fork arrest (Bastia et al., 2008).

Resolution of daughter DNA molecules at Ter sites: Following fork arrest at *Ter* sites, the daughter DNA molecules are resolved by a special type II topoisomerase, namely Topo IV (Espeli et al., 2003). It has been reported that this topoisomerase is stimulated by the actin-like MreB protein that acts near the resolution site *dif* that resolves dimers generated by recombination (Madabhushi and Marians, 2009).
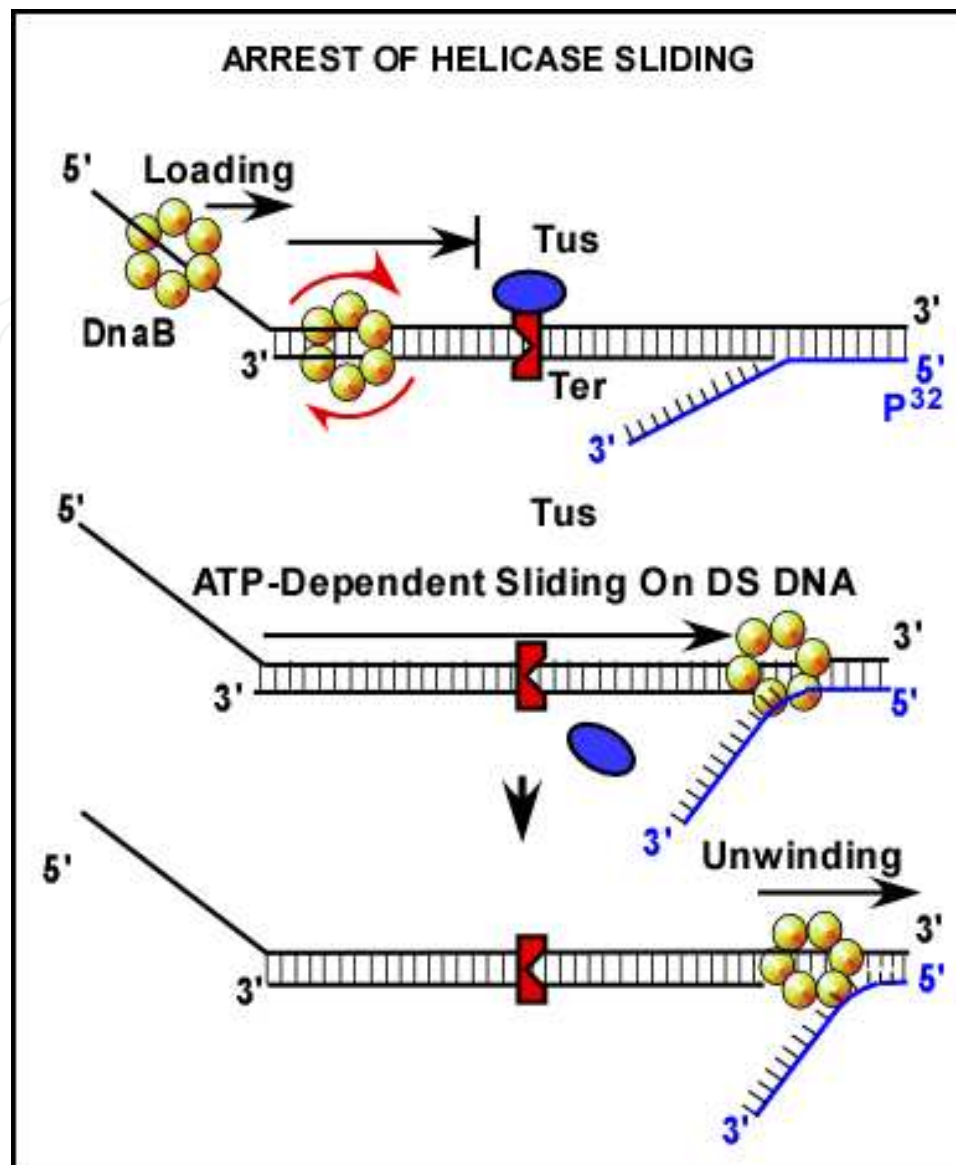
Fig. 4. A substrate designed to separate temporally and spatially DnaB translocation from DNA unwinding. A 5′ tailed DNA with otherwise a blunt end on the complementary strand enters the substrate and then slides over the dsDNA until it meets the fork like structure (in blue) and unwinds the labeled strand. If a Tus-*Ter* complex is present in a blocking orientation, the sliding DnaB is arrested, thereby preventing the unwinding of the blue strand; a *Ter* site in the permissive orientation when bound to Tus displaces Tus and slides down the substrate and unwinds the blue strand. The results showed that DnaB sliding, without any DNA melting was arrested in a polar mode by the Tus-*Ter* complex, thereby showing that DNA unwinding (and presumably base flipping) is not necessary for polar helicase/ fork arrest.

Replication termini in eukaryotes: Many, perhaps all, eukaryotes have sequence-specific replication termini located in their ribosomal DNA (rDNA) array. For example, *Saccharomyces cerevisiae* contains a pair of Ter sites in one of the nontranscribed spacers of each rDNA unit between the sequences encoding the 35S RNA and the 5S RNA (Brewer and Fangman, 1988; Brewer et al., 1992; Ward et al., 2000). The second spacer contains a

replication *ori* (*ars*; see Fig.5). The Ter sites bind to the replication terminator protein called Fob1 (fork blockage) (Kobayashi, 2003; Kobayashi and Horiuchi, 1996; Mohanty and Bastia, 2004). The Fob1 protein bound to Ter sites prevents replication forks moving from right to left from colliding with the strong transcription of 35S RNA. It has been shown that transcription-replication collision causes not only fork stalling but also stalled RNA polymerase and an incomplete RNA transcript that can hybridize with DNA to form an R loop. R loops, especially the single stranded DNA therein, is susceptible to physical and enzymatic damage *in vivo* which causes genome instability (Helmrich et al., 2011).
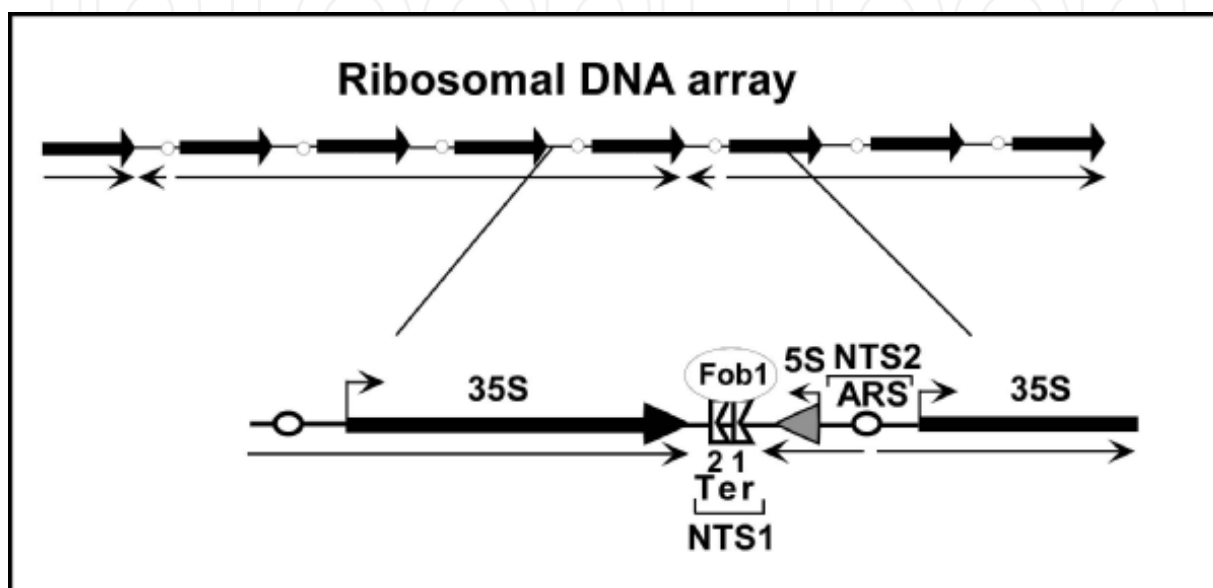

Fig. 5. rDNA repeat region in chromosome XII of *S. cerevisiae* showing the location of the two Ter sites in the nontranscribed spacer 1 (NTS1). The replication is initiated bidirectionally from the *ars* present in nontranscribed spacer 2 (NTS2). The Ter sites prevent replication forks moving to the left from the *ars* from running into RNA polymerase transcribing the 35S rRNA precursor.

The Fob1 protein is multifunctional and loads histone deacetylase to silence intra-chromatid recombination in the tandem array of ~200 rDNA repeats that might otherwise lead to unscheduled loss or gain of rDNA repeats (Bairwa et al., 2010; Huang et al., 2006; Huang and Moazed, 2003). Fob1 protein is also a transcriptional activator and controls exit from mitosis (Bastia and Mohanty, 2006; Stegmeier et al., 2004).

One of the facile techniques to study Fob1 function is to perform segment-directed mutagenesis, which is shown schematically (Fig.6). A segment of an ORF flanked by regions of homology (also from the ORF) is amplified by PCR under conditions of low fidelity synthesis in which one of the dNTPs is present at a suboptimal concentration. This leads to misincorporation of the base into DNA causing random mutations. A plasmid containing a gap corresponding to the segment being mutagenized and the PCR products are used to transform yeast. The mutagenized DNA segment gets incorporated into the plasmid by gap repair caused by the homologous recombination machinery of yeast with high efficiency, thus generating a pool of potential mutants contained in the plasmid. The plasmid contains a marker expressed in yeast (*e.g.*, *Leu*) and an *ars*. Using this protocol, we extensively mutagenized Fob1 and were able to identify many of its functional domains, such as its

DNA binding domain and a domain for its interaction with the silencing linker protein called Net1. Net1 recruits the histone deacetylase Sir2 onto Fob1 by direct protein-protein interaction between Net1 and Sir2 on one hand and between Net1 and Fob1 on the other, and loads Sir2 near the Ter sites. This process, as noted above, causes silencing of rDNA and prevents unwanted recombination (Bairwa et al., 2010; Mohanty and Bastia, 2004). At this time, the detailed mechanism of replication termination in eukaryotes has not been elucidated. However, it is known that two intra-S checkpoint proteins called Tof1 and its interacting partner called Csm3 are necessary for stable fork arrest at Ter because the Tof1-Csm3 complex protects the Fob1 protein from getting displaced from the Ter site by the action of the helicase Rrm3 (Mohanty et al., 2006, 2009). The catenated daughter molecules at Ter sites in *S. cerevisiae* are separated from each other by Topo II (Baxter and Diffley, 2008; Fachinetti et al., 2010).
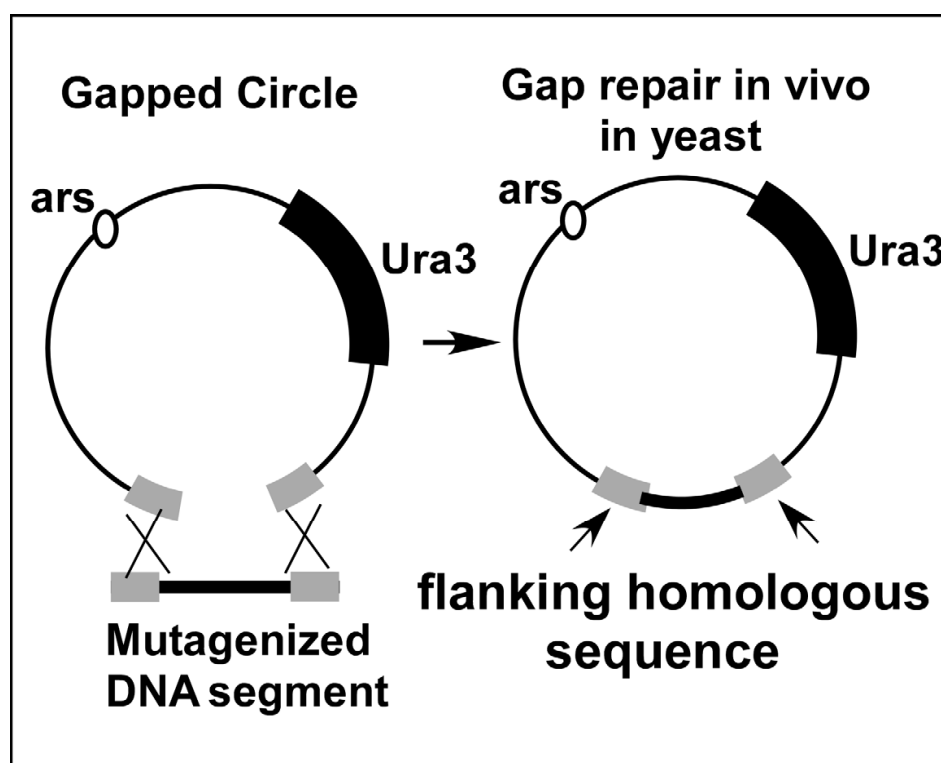

Fig. 6. Schematic diagram showing segment-directed mutagenesis and recovery of mutants by gap repair. The gapped plasmid is prepared by restriction site cutting inside the ORF. The DNA segment is mutagenized by low-fidelity PCR that includes primers with homologous flanking sequence. Transformation of a mixture of mutagenized DNA mixed with the gapped plasmid results in a pool of plasmids, some of which should have random base changes within the mutagenized DNA segment

We have recently reported that the Reb1 terminator protein binding to 2 Ter sites of fission yeast act in a cooperative fashion. The dimeric Reb1 protein, for example, brings into contact a Ter site located on chromosome 2 with two Ter sites located on chromosome 1. Interestingly there was no interaction observed between sites on chromosome 1 and 2 with the Ter sites located in the two rDNA clusters present on chromosome 3. It seems that the Ter-Ter interactions are not random. We further reported that the interactions called "chromosome kissing' modulated the activities of the Ter sites (Singh et al., 2010).

Physiological function of the replication termini: In prokaryotes, the replication termini perform at least 2 functions: (i) these serve as a replication trap and confine the meeting of the two approaching forks to the *TerC* region (Fig.1) where the dimer resolution (*dif*) sites are located. This activity probably facilitates chromosome segregation (Wake, 1997); and (ii) the terminus, in plasmid chromosomes prevents accidental switch to a rolling circle mode of replication that would generate unwanted linearly catenated chromosome (Dasgupta et al., 1991). In eukaryotes, the termini probably serve as barriers to transcription-replication collision that might generate destabilizing R loops. The termini are also known to be involved in cellular differentiation of fission yeast (Dalgaard and Klar, 2000, 2001). As noted above, Fob1 protein has diverse other functions (Bastia and Mohanty, 2006; Kaplan and Bastia, 2009).

In summary, replication termination at site-specific termini is an important part of DNA replication that invites further investigation, especially in eukaryotes, because of its role in various DNA transactions including maintenance of genome stability.

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