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RecBCD, SbcCD and ExoI process a substrate created by convergent replisomes to complete DNA replication

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Summary

The accurate completion of DNA replication on the chromosome requires RecBCD and structure specific SbcCD and ExoI nucleases. However, the substrates and mechanism by which this reaction occurs remains unknown. Here we show that these completion enzymes operate on plasmid substrates containing two replisomes, but are not required for plasmids containing one replisome. Completion on the two-replisome plasmids requires RecBCD, but does not require RecA and no broken intermediates accumulate in its absence, indicating that the completion reaction occurs normally in the absence of any double-strand breaks. Further, similar to the chromosome, we show that when the normal completion reaction is prevented, an aberrant RecA-mediated recombination process leads to amplifications that drive most of the instabilities associated with the two-replisome substrates. The observations imply that the substrate SbcCD, ExoI and RecBCD act upon *in vivo* is created specifically by two convergent replisomes, and demonstrate that the function of RecBCD in completing replication is independent of double-strand break repair, and likely promotes joining of the strands of the convergent replication forks.

Introduction

Cells tightly regulate DNA replication initiation, elongation and completion to ensure that each daughter cell inherits an identical copy of the genetic information. While the mechanisms regulating initiation and elongation have been well characterized (reviewed in Costa *et al.* (2013)), the process of how cells recognize replicated regions and complete replication at the precise point where all sequences have doubled has until recently, remained unknown. To complete replication accurately, cells must encode an enzymatic system that is capable of recognizing or counting in pairs, and joins the strands of converging replication forks at the point where all sequences have precisely doubled. The failure to complete a single replication event would be expected to result in a loss of genomic stability, mutation or cell lethality. Yet, this reaction occurs thousands of times per generation along the chromosomes of human cells, and therefore, must occur with remarkable efficiency. Given this critical role, and considering the large number of proteins that cells devote to ensure fidelity during replication initiation and elongation, it is not surprising that this final step is also tightly regulated and controlled enzymatically (Rudolph *et al.*, 2013; Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015; Dimude *et al.*, 2015; Dimude, Midgley-Smith *et al.*, 2018; Dimude, Stein *et al.*, 2018; Midgley-Smith *et al.*, 2018).

A number of studies demonstrate that an ability to sense when all sequences in the genome have doubled is critical to genomic replication. *In vitro,* converging replisomes continue through their meeting point as one replisome displaces the other, resulting in over-replication, or a third copy, of the region where the forks meet (Hiasa and Marians, 1994). Over-replication also occurs *in vivo* and is prominently observed on the chromosome of cells lacking the helicases and exonucleases required to disrupt and degrade these events (de Massy, Fayat *et al.*, 1984; de Massy, Patte *et al.*, 1984; Asai *et al.*, 1994; Rudolph, Upton, & Lloyd, 2009; *et al.*, 2013; Wendel *et al.*, 2014; 2018; Dimude *et al.*, 2015; Dimude *et al.*, 2016; Dimude, Midgley-Smith *et al.*, 2018; Midgley-Smith *et al.*, 2018). Other studies suggest that illegitimate initiations of replication associated with repair events or transcriptional processes occur frequently at single strand nicks, gaps, D-loops and R-loops throughout the genomes of both prokaryotes and eukaryotes (de Massy, Fayet *et al.*, 1984; Magee *et al.*, 1992; Asai and Kogoma, 1994; Donnianni and Symington, 2013; Bhatia *et al.*, 2014; Hamperl and

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Cimprich, 2014; Brochu *et al.*, 2018). Each of these events would generate a third copy of the chromosomal region where the replication initiates. Thus, over-replication appears to be an inherent and promiscuous problem during the duplication of genomes. These considerations make it clear that enzymes catalyzing the accurate and efficient completion of replication are essential to genomic stability and are likely required for cellular life overall.

The completion step of DNA replication has been challenging to study in eukaryotic cells, in part because multiple origins are utilized with varying efficiencies and timing, making the regions where forks meet highly variable between cells and cell cycles (Heichinger *et al.*, 2006; Wu and Nurse, 2009). By comparison, *Escherichia coli* is uniquely suited to dissect this fundamental aspect of cellular metabolism since the event can be localized to a single ~400 kb region of the chromosome, opposite to its bidirectional origin of replication (reviewed in (Hill, 1992)). This region is flanked by *ter* sequences which bind the protein Tus, blocking replication forks in an orientation specific manner (Kobayashi *et al.*, 1989). Although *ter* ensures that completion occurs within this region, it is not involved in the reaction, as chromosomes lacking *ter* replicate normally and are stably maintained (Roecklein *et al.*, 1991; Duggin *et al.*, 2008; Duggin and Bell, 2009).

Current models of completion in *E. coli* suggest that converging replisomes transiently bypass each other at the point where they converge, creating an over-replicated region that contains three copies of the genetic information (Fig. 1). The completion reaction is thought to initiate through the action of SbcCD and ExoI structure specific nucleases which act on a structural intermediate created at the point where forks converge (Dimude, Midgley-Smith *et al.*, 2018; Wendel *et al.*, 2018). In the absence of the SbcCD and ExoI nucleases, the over-replicated region persists, leading to genomic instabilities and amplifications at these loci (Rudoloph *et al.*, 2013; Wendel *et al.*, 2014; 2018; Dimude, Midgley-Smith *et al.*, 2018). Following incisions by these enzymes, the RecBCD helicase-nuclease complex processes the over-replicated intermediate and is required to catalyze or recruit enzymes that promote joining of the convergent strands. In vitro, RecB and RecC interact with RecD to form a dual helicase–nuclease complex that unwinds and degrades double-strand DNA ends (Taylor and Smith, 1985; Amundsen *et al.*, 1986; Taylor and Smith, 2003). Loss of RecB or -C inactivates the enzyme complex, whereas loss of RecD inactivates the nuclease, but retains the helicase activity and recombination proficiency of the complex (Taylor and Smith, 1985; Amundsen *et al.*, 1986; Taylor and Smith, 2003). On the chromosome, in the absence of RecB or C, the nascent ends of convergent replication forks are not joined, leading to excessive degradation and rendering cells unable to maintain the chromosome region where forks converge (Rudolph *et al.*, 2013; Wendel *et al.*, 2014; 2018). The inability to complete replication or maintain these regions of the genome severely compromises the viability and growth of *recBCD* cultures (Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015). In the absence of *recD*, degradation of the

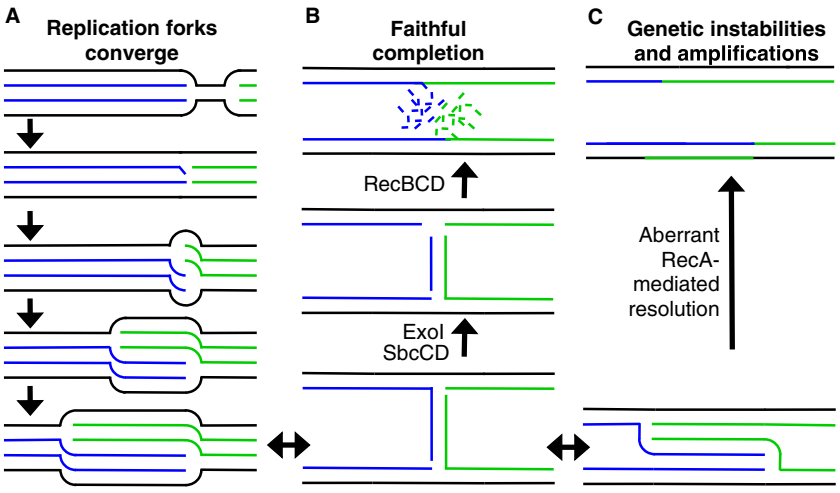

Fig. 1. Current model for completing DNA replication.
A. Convergent replication forks continue past their meeting point, creating a partially over-replicated substrate that contains three copies of the genetic information.
B. SbcCD-ExoI recognize and cleave this branched over-replicated substrate which creates a DNA that can be resect and processed by RecBCD. Following resection, RecBCD promotes or recruits enzymes that join the convergent strands at the doubling point.
C. When processing by SbcCD-ExoI is prevented or impaired, the over-replicated region persists, and an aberrant form of recombination then resolves the over-replicated region, leading to amplifications and genomic instabilities. [Colour figure can be viewed at wileyonlinelibrary.com]

excess sequence is impaired, however, joining appears to occur normally and viability is not compromised (Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015).

The completion reaction can occur normally in the absence of RecA or recombination (Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015). However, when completion is impaired or prevented from processing the over-replicated intermediates, viability and growth become dependent on an aberrant form of RecA-mediated recombination that leads to genetic instabilities and amplifications of these loci (Wendel *et al.*, 2018). Similar instabilities and amplifications are observed in eukaryotes lacking the homologs Mre11-Rad50 and Sae2, suggesting that the completion reaction is one that is conserved throughout evolutionarily diverged organisms (Lengsfeld *et al.*, 2007; Bruhn *et al.*, 2014; Deng *et al.*, 2015).

Here, we used plasmid mini-chromosomes to further characterize the completion reaction. We show that SbcCD, ExoI and RecBCD are required to propagate substrates containing two replisomes but not one replisome, suggesting that the substrates acted upon by these enzymes is specific to a structure created when two replisomes converge. We further show that similar to completion sites on the chromosome, genetic instability on plasmids containing two replisomes are driven by amplifications associated with an aberrant, RecA-mediated recombinational reaction.

Results

Construction and characterization of plasmids replicated by two replisomes

To further characterize the completion of replication reaction, we utilized a plasmid containing a bidirectional origin of replication that would allow us to characterize the genes and sequences associated with maintaining a minichromosome that contains convergent replication forks (Fig. 2A). Plasmid pCL01 contains a lambda origin of replication, which loads dual helicases and utilizes the host's replication proteins (reviewed in Stillman (1994)). For the purposes of comparison and control, we compared the replication and stability of these two-replisome plasmids to pBR322, a well-characterized plasmid that maintains a moderate copy number and also utilizes the host's replication machinery, but has a unidirectional ColE1 origin of replication and propagates using a single replisome (Martin-Parras *et al.*, 1991).

The two-replisome plasmid was stably propagated in the presence of selection. However, when grown without selection, the two-replisome plasmids was far less stable than the one-replisome plasmid (Fig. 2B). Whereas the unidirectionally replicating pBR322 was stably maintained without loss over 30 generations, only 0.1–1.0% of cells maintained the bidirectionally replicating plasmids over the same duration.

The reduced stability of the two-replisome plasmid, relative to the one-replisome plasmid, was not due to growth rate inhibition or a lower overall copy number. Cultures containing no plasmid, one-replisome plasmids, or two-replisome plasmids all grew at similar rates (Fig. 2C). To examine plasmid copy number, total genomic DNA was purified from cultures containing the ampicillin resistance gene integrated into the chromosome, as well as cultures containing the one-replisome and two-replisome plasmids, which also contain this gene. The purified DNA was then digested with a restriction enzyme that linearizes the plasmid before DNA from equal cell equivalents was analyzed by Southern analysis following agarose-gel electrophoresis using a ^{32}P-labeled ampicillin resistance gene as a probe. As shown in Fig. 2D and E, both the one-replisome and two-replisome plasmids were maintained at similar copy numbers and ranged between 50-80 copies per chromosome. These numbers are similar to those previously reported for pBR322 and plasmids containing a phage lambda origin (Boros *et al.*, 1984; Lupski *et al.*, 1986; Boyd and Sherratt, 1995; Atlung *et al.*, 1999).

These initial observations indicate that the two-replisome plasmid, pCL01, can replicate and propagate in cells, but that it is less stable than the one-replisome plasmid, pBR322. However, the reduced stability is not due to an effect on the growth rate of cells containing this plasmid or a lower copy number, relative to pBR322. Further, neither plasmid contains partitioning genes or sequences that would account for the difference in stability.

Plasmids with two-replisomes, but not one-replisome, require enzymes needed to complete replication on the chromosome

We next asked whether the genes required to complete replication on the chromosome were also needed to maintain the two-replisome plasmids. Completion of replication on the chromosome requires RecB and -C to resect and join the over-replicated region where forks converge. In their absence, joining does not occur and cells are unable to maintain these regions of their genome (Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015) (Fig. S1). Similarly, we found that the ability of cells to propagate plasmids containing two-replisomes was severely impaired in the absence of RecBC. Transformation of the two-replisome plasmid was reduced by more than two orders of magnitude in *recBC* mutants (Fig. 3). By comparison, the absence of RecD does not impair the cell's ability to maintain regions where completion occurs, and transformation of the two-replisome plasmid was not reduced in *recD* mutants. The impaired ability to transform *recBC*

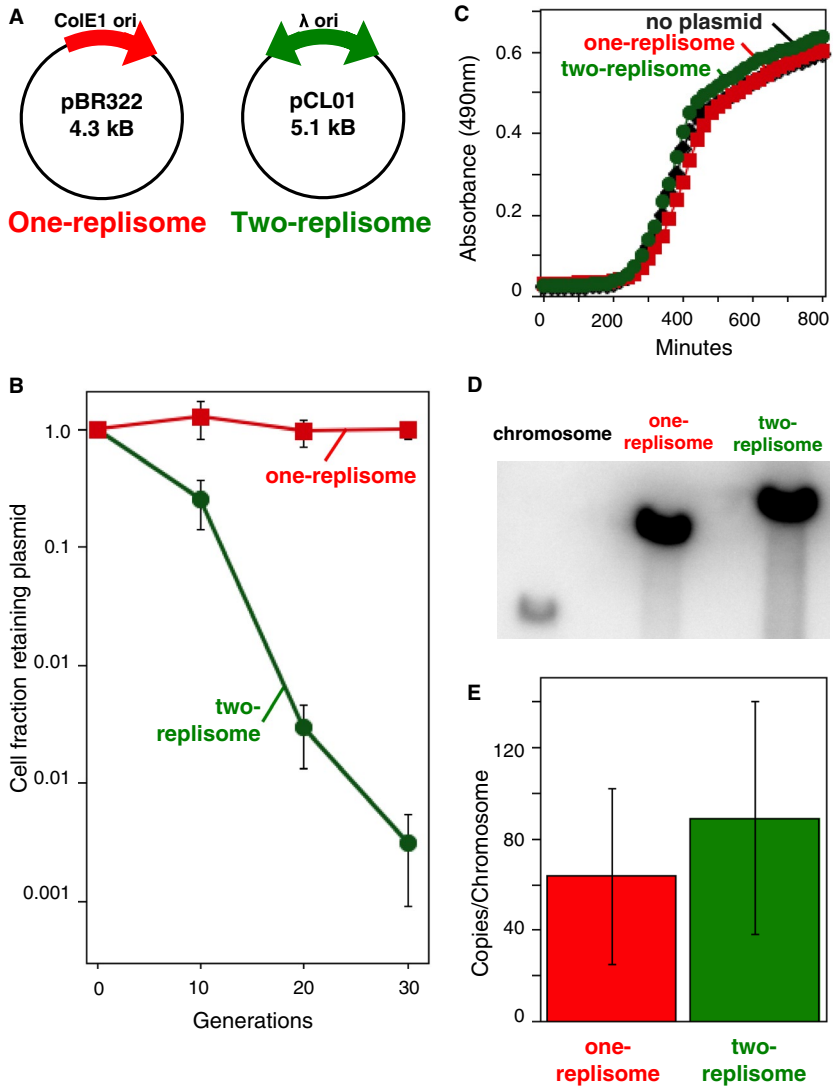

Fig. 2. Plasmids replicated by two-replisomes are less stable than those replicated by one-replisome.
A. *Diagram of the one-replisome and two-replisome plasmids, containing a ColE1 and lambda origin of replication, respectively.*
B. *In the absence of selection, the two-replisome plasmid is lost more rapidly than the one-replisome plasmid.* Cultures containing the one-replisome (pBR322) or two-replisome (pCL01) plasmid were grown without selection. Serial dilutions of the culture were then plated with and without ampicillin selection to determine the fraction of cells that retained the plasmid over time. Error bars represent the standard error of four or more independent experiments.
C. *The instability of the two-replisome plasmid, relative to the one-replisome plasmid is not due a reduced growth rate of cells containing the two-replisome plasmid.* The absorbance at 630 nm of cultures containing no plasmid, pBR322 or pCL01 grown at 37°C is plotted over time.
D. No plasmid (diamonds); pBR322 (squares); pCL01 (circles). *The instability of the two-replisome plasmid, relative to the one-replisome plasmid is not due to a reduced copy number during growth.* A representative Southern analysis of the ampicillin resistance gene on the chromosome, on the plasmid pBR322, and on the plasmid pCL01 is shown. Total genomic DNA was purified from HL946 (Courcelle *et al.*, 1999), containing a chromosomal copy of the ampicillin resistance gene, SR108 containing pBR322 and SR108 containing pCL01. Purified DNA was digested with EcoRV to linearize the plasmids and equal cell equivalents were then loaded and analyzed by Southern analysis using a ^{32}P-labeled ampicillin resistance gene as a probe.
E. *The copy number of each plasmid, relative to the chromosome is plotted.* Error bars represent the standard error from five independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

mutants was specific to the plasmid with two replisomes, as one-replisome plasmids, pBR322 and pREP4, were transformed in *recBC* mutants at frequencies similar to wildtype cultures (Figs 3 and S2). The observation suggests that RecBC is specifically required to process a structure created when two replisomes converge.

If the impaired ability of two-replisome plasmids to transform *recBC* mutants were due to defective repair of double-strand breaks that arise on these plasmid substrates, then one would expect that mutants lacking RecA, which is essential for double-strand break repair, to exhibit a similar deficiency. However, unlike

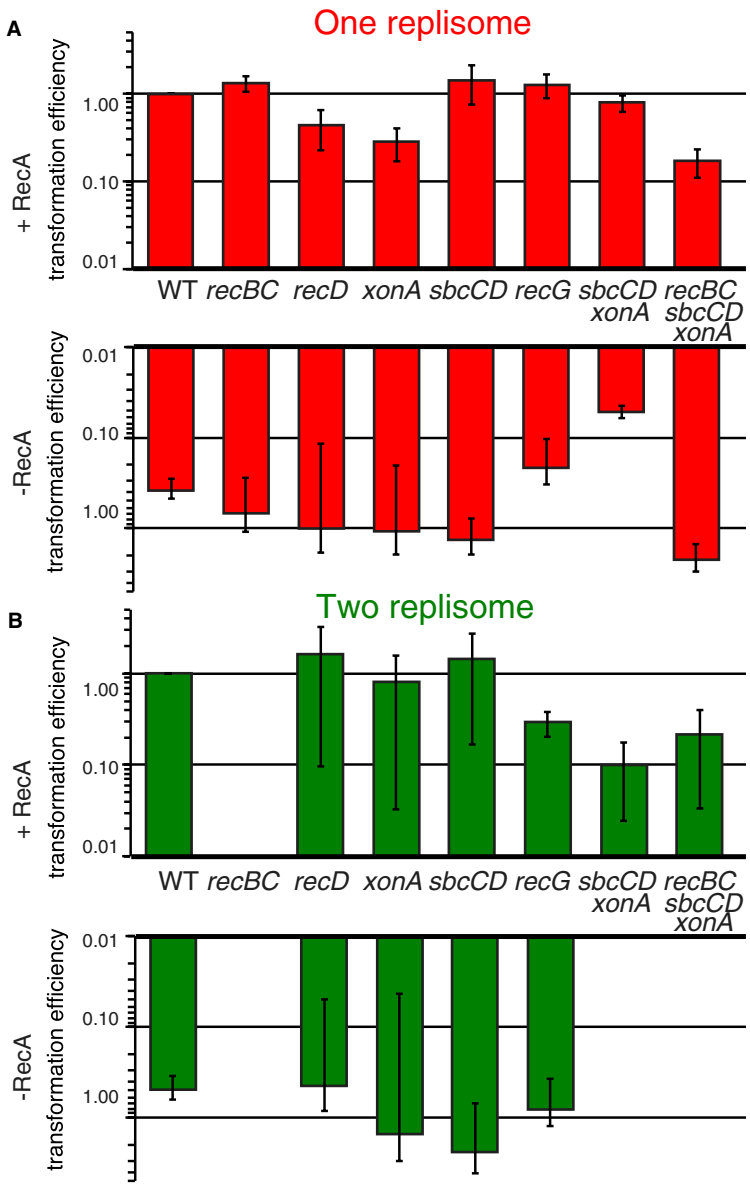

Fig. 3. Transformation of plasmids with two replisomes, but not one replisome, depends on the enzymes required to complete replication on the chromosome.
A. The transformation efficiency relative to wildtype cells is shown for the strains indicated following electroporation of competent cells with 50 ng of pBR322 (one replisome).
B. The transformation efficiency relative to wildtype cells is shown for the strains indicated following electroporation of competent cells with 50 ng of pCL01 (two replisomes). Error bars represent the standard error of at least two independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

recBC mutants, the two-replisome plasmid transformed *recA* mutants at frequencies comparable to wildtype cells. Thus, similar to what is observed on the chromosome, the RecBC-mediated completion reaction on the plasmid does not require recombination or RecA, and is unlikely to be associated with a double-strand break-intermediate.

On the chromosome, the completion reaction is initiated by the SbcCD and ExoI nucleases, encoded by *sbcC*, *sbcD* and *xonA* (also called *sbcB*. Kushner *et al.* (1972)). Together, these enzymes are required to incise and/or resect the DNA structure created at sites where convergent replication forks meet (Wendel *et al.*, 2018). When these nucleases are inactivated, the over-replicated region where replication forks converge persists, preventing replication from completing normally. Under these conditions, the ability to maintain these regions and continue to grow becomes dependent on an aberrant RecA-mediated recombinational process that results in genomic instabilities and amplifications at sites

where forks converge (Wendel *et al*., 2018) (Fig. S1). To determine if the two-replisome plasmids also rely upon these enzymes, we compared the ability of the two-replisome plasmid to transform *sbcCD xonA* mutants, both in the presence and absence of RecA. We found that strains lacking the SbcCD and ExoI nucleases could be transformed by the two-replisome plasmid. However, in the absence of these gene products, transformation depended on the presence of RecA. The observations are consistent with the idea that, similar to the chromosome, when the normal mechanism of completion is impaired or inactivated, the reaction is shunted through a recombinational mechanism that depends on RecA (Wendel *et al*., 2018). Additionally, RecA-dependence of *sbcCD xonA* mutants was specific to plasmids containing two replisomes, as the single-replisome plasmid, pBR322, could successfully transform *sbcCD xonA* mutants in the presence or absence of RecA (Fig. 3A).

For comparison, we also examined mutants lacking RecG. On the chromosome, over-replicated regions persist in *recG* mutants, similar to *sbcCD xonA* mutants. However, the over-replication in *recG* mutants is thought to arise from a failure to disrupt illegitimate replication initiation events from R- and D-loops (Rudolph, Upton, Harris *et al*., 2009; Rudolph, Upton, and Lloyd, 2009; Rudolph *et al*., 2010; Rudolph *et al*., 2013; Dimude *et al*., 2016; Lloyd and Rudolph, 2016; Midgley-Smith *et al*., 2018), rather than a direct involvement in the completion reaction (Wendel *et al*., 2014). We found that the two-replisome plasmid transformed *recG* mutants both in the presence or absence of RecA, arguing that the RecA dependence does not extend to all mutants exhibiting an over-replication phenotype.

Similar to the chromosome, the effect of the *sbcCD* and *xonA* mutations were additive (Wendel *et al*., 2018), as the absence of either gene product alone did not prevent the ability to maintain the two-replisome plasmid in the absence of RecA. Additionally, inactivation of both SbcCD and ExoI restored the ability of *recBC* mutants to transform and maintain the bidirectional plasmid through a mechanism that depended upon RecA (Fig. 3B), consistent with the idea that without initiation of the completion reaction by SbcCD and ExoI, the downstream function of RecBCD becomes unnecessary, as the reaction is shunted to the aberrant RecA-mediated pathway.

These observations are all consistent with the mechanisms that complete replication on the chromosome and suggest that the two-replisome plasmid may serve as a useful model to dissect the intermediates involved in this reaction. Additionally, requirement for the completion enzymes on the two-replisome plasmid, but not the one-replisome plasmid argues that the substrate RecBCD, SbcCD and ExoI act upon in vivo is specifically created when two replisomes converge.

The aberrant recombinational pathway is responsible for the instability of plasmids with two replisomes

The results above show that transformation of two-replisome, but not one-replisome plasmids depends on the factors needed to complete chromosome replication and that plasmids containing two replisomes exhibit instability relative to those containing one replisome, On the chromosome, when the normal mechanism of completion is impaired or prevented, the reaction occurs through an aberrant RecA-mediated pathway that is associated with genomic instabilities and amplifications in this region (Wendel *et al*., 2018).

To examine if recombination played a role in the instability associated with the two-replisome plasmid, we examined the ability of *recA* mutants to maintain these plasmids in the absence of selection. We found that most of the instability on the two-replisome plasmid was due to RecA and was associated with the appearance of amplifications and aberrant plasmid species. Inactivation of RecA markedly increased the stability of the two-replisome plasmid (Fig. 4A). The increase in stability of the *recA* mutant containing the two-replisome plasmids approached that seen for the one-replisome plasmid, indicating that most of the instability on the two-replisome plasmid was driven by recombinational mechanisms. We next examined the form of these plasmids in cells. To this end, total genomic DNA was purified from cultures containing the plasmid and examined by Southern analysis using a ^{32}P-labeled plasmid as the probe. We observed that, relative to the one-replisome plasmid, the two-replisome plasmid contained a higher proportion of amplifications and aberrant multimeric species (Fig. 4B and C). However, in the absence of *recA*, the proportion of aberrant multimeric plasmid species noticeably diminished and a higher proportion of the molecules were maintained as monomeric circles (Fig. 4B and C).

We additionally examined several other mutants, including *recD*, *recG* and *sbcCD xonA*, to determine if the absence of other recombination enzymes would increase the stability of the two-replisome plasmid similar to *recA*. None of the mutants examined improved the stability to the two-replisome plasmid relative to wildtype cells (Fig. 5A). We were unable to examine *recBC* mutants due to their impaired ability to transform or stably maintain the two-replisome plasmid. We also examined the form of the plasmid in these mutants and found that the mutations also did not reduce the overall proportion of aberrant multimeric plasmid species that were observed (Fig. 5B). In some preparations, a large fraction of the plasmid DNA in *recD* and the *recBC sbcCD xonA* strains migrated as high-molecular-weight multimers (Fig. S4). This also occurs on one-replisome plasmids in these strains and reduces the stability of

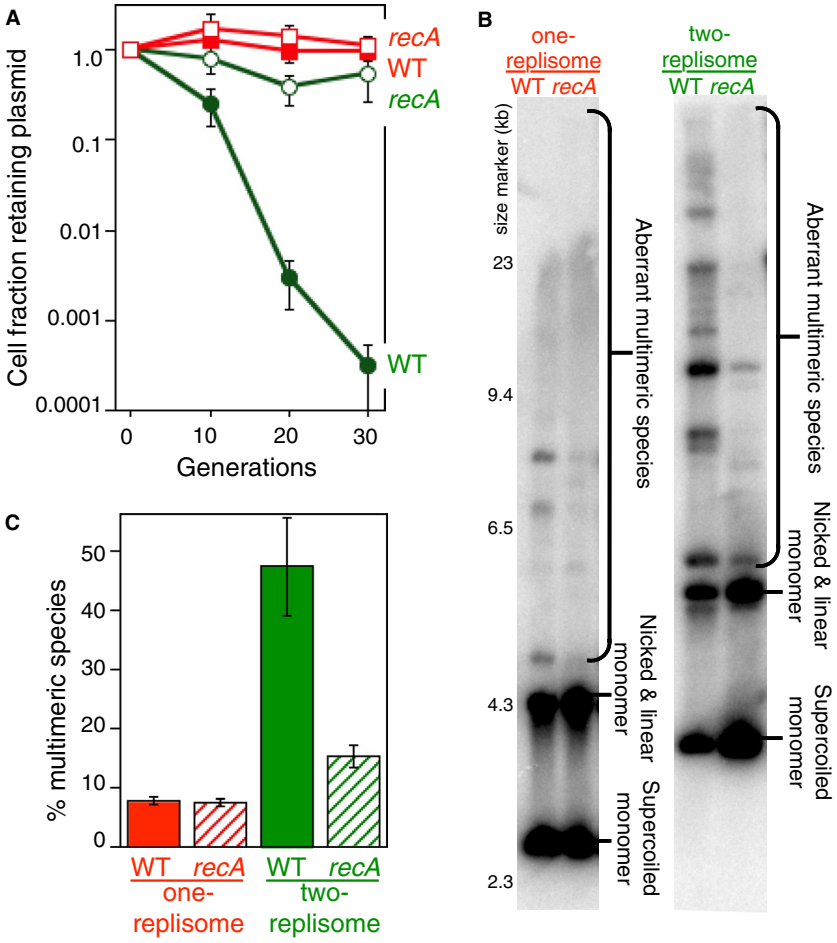

Fig. 4. Similar to completion on the chromosome, amplifications and instability on two-replisome plasmids are driven by an aberrant RecA-mediated form of recombination.
A. *Inactivation of RecA restores the stability of the two-replisome plasmid to levels that approach that of the one-replisome plasmid.* Cultures were treated as in Fig. 2B. The fraction of cells retaining the one-replisome and two-replisome plasmid in wildtype (WT) and *recA* mutants is plotted over time. Error bars represent the standard error of four independent experiments. WT pBR322 (filled squares); *recA* pBR322 (open squares); WT pCL01 (filled circles); *recA* pCL01 (open circles).
B. *Plasmid instability promoted by RecA correlates with an increased level of amplifications and aberrant multimeric species*. Total genomic DNA from cells containing the one-replisome or two-replisome plasmid was purified and analyzed by Southern analysis following agarose gel electrophoresis using ^{32}P-labeled pBR322 or pCL01 as a probe.
C. *The fraction of unit-length monomeric plasmid is plotted for the one-replisome and two-replisome plasmid in the presence and absence of RecA*. Error bars represent the standard error of four independent experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

these plasmids overall (Seelke *et al.*, 1987; Silberstein and Cohen, 1987; Niki *et al.*, 1990; Wendel *et al.*, 2014; 2018). On the two-replisome plasmid, the high proportion of multimeric species did not appear to further reduce the plasmid loss rate beyond the relatively high rate already observed in wildtype cells. However, we noted that in all strains where the plasmid was unstable, there was significant variation observed between the total and proportion of plasmid observed in strains during analysis (Fig. S4).

Taken together, Fig. 4 and 5 argue that a RecA-mediated recombinational reaction is responsible for the amplifications and aberrant species on the two-replisome plasmid, and that these products are driving the instability of these plasmids, similar to that seen at sites where replication completes on the chromosome.

Discussion

Here, we show that maintaining plasmids containing two replisomes depends on the enzymes needed to complete replication on the chromosome. On the chromosome, the completion of replication requires RecBCD to join the strands of convergent replication forks on the chromosome. In its absence, DNA ends persist, are extensively degraded, and cells fail to maintain these regions of the

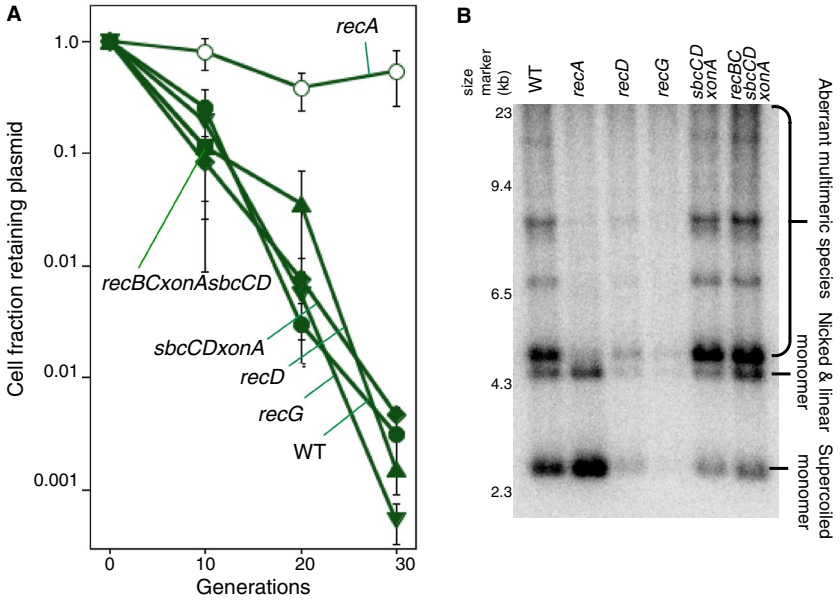

Fig. 5. Stability of the two-replisome plasmid in various mutants.
A. *The fraction of cells retaining the two-replisome plasmid for each strain is plotted over time*. Cultures were treated as in Fig. 2B. WT (filled circles); *recA* (open circles); *recD* (filled triangles); *recG* (filled inverted triangles); *sbcCD xonA* (diamonds); *recBC sbcCD xonA* (filled squares); Error bars represent the standard error of at least three independent experiments.
B. *A representative Southern analysis of plasmid pCL01 grown in each strain is shown*. DNA was purified and analyzed as in Fig. 4B. [Colour figure can be viewed at wileyonlinelibrary.com]

chromosome (Rudolph *et al*., 2013; Wendel *et al*., 2014; 2018; Courcelle *et al*., 2015; Dimude, Midgley-Smith *et al*., 2018). Similarly, we show that transformation of two-replisome plasmids in *recBC* mutants is severely impaired and the plasmids fail to propagate in cells under selection. On the chromosome, the SbcCD and ExoI structure-specific nucleases are required to initiate the faithful completion reaction. In the absence of these proteins, normal completion cannot occur, excess over-replicated regions persist and the reaction is shunted through a RecA-mediated pathway that is associated with amplifications and genetic instabilities (Rudolph *et al*., 2013; Wendel *et al*., 2014; 2018; Courcelle *et al*., 2015; Dimude, Midgley-Smith *et al*., 2018). Here, we show that transformation of two-replisome plasmids in *sbcCD xonA* mutants similarly depends on the RecA-mediated pathway. Thus, both the normal *recBCD*-mediated reaction, and the aberrant recombinational process appear to operate on the two-replisome plasmids. Additionally, we show that the requirement for these completion enzymes is specific to plasmids with two-replisomes, as transformation was not impaired in the one-replisome plasmid, pBR322. The observation implies that the substrate acted upon by SbcCD, ExoI and RecBCD during the completion reaction is specific to a structure created when two replisomes converge.

Finally, we found that the two-replisome plasmid was less stable than the one-replisome plasmid when propagated in the absence of selection, and showed that this instability was driven by amplifications arising from a RecA-mediated recombination reaction, similar to what is observed on the chromosome at sites where completion occurs (Wendel *et al*., 2018). Inactivation of RecA increased the stability of the two-replisome plasmid and reduced the proportion of abnormal amplification products that were observed (Fig. 5). The high rate of RecA-driven instability on the two-replisome plasmid indicates that the aberrant pathway occurs at relatively high frequency on the plasmid, even when the normal, faithful pathway remains functional. We would speculate that this is likely due to the high copy number of the plasmid, and that the frequency of aberrant completion events on the chromosome is likely to be much less. Under normal conditions in *E. coli,* the completion enzymes are only required to catalyze a single reaction on the chromosome. Whereas the bi-directional plasmid, which is maintained at ~80 copies/chromosome (Fig. 2), may exceed the reaction capacity of the faithful pathway, whose genes are not highly expressed (Eichler and Lehman, 1977; Taylor and Smith, 1980), allowing the aberrant mechanism to operate more frequently than would normally occur.

To our knowledge, examples of other well-characterized plasmids with bi-directional origins have not been reported. Plasmid R6K was originally reported to have a bidirectional origin (Lovett *et al*., 1975), but subsequent work found the plasmid contained two unidirectional origins and employed a complex asymmetric mechanism of replication (Crosa *et al*., 1976). In our initial approach to this work, we initially utilized a plasmid containing

the *E. coli* origin of replication, pOriC-2 (Sawitzke *et al.*, 2012). However, consistent with previous studies on *oriC* plasmids, we found that the extensive homology with the chromosome led to high frequency integration events (Yasuda and Hirota, 1977; Messer *et al.*, 1978; Meijer *et al.*, 1979). These frequent integration events prevented a direct analysis of plasmid stability, as cultures with integrated plasmids maintained resistance to ampicillin even after the extrachromosomal plasmid itself was lost (Fig. S3).

ExoI and SbcCD nuclease complexes are additive in their effect on the completion reaction and inactivation of the normal completion reaction required deletion of both genes (Wendel *et al.*, 2014; 2018; Dimude, Midgley-Smith *et al.*, 2018). On the chromosome, in the absence of both nucleases, the over-replicated regions persist and the ability to maintain growth and the chromosome region where forks converge becomes entirely dependent on RecA (Wendel *et al.*, 2018). In this report, we show that transformation of two-replisome plasmids similarly becomes dependent on RecA only in the absence of both nucleases (Fig. 3). Mechanistically, SbcCD and ExoI could functionally interact to cooperate and enhance their ability to degrade the substrate(s) created by convergent replication forks. This appears to occur in eukaryotes, where the homologous Mre11-Rad50 interacts with Sae2 and increases its exonuclease activity (Lengsfeld *et al.*, 2007; Deng *et al.*, 2015; Andres and Williams, 2017). Consistent with this, a recent study from Dimude et al. found that SbcCD alone prevented much of the degradation that occurs in *recBC* mutants (Dimude, Midgley-Smith *et al.*, 2018), an observation we have confirmed in our lab. In vitro, SbcCD has been demonstrated to cleave a palindrome-like substrate similar to that predicted to occur when replication forks bypass each other (Lim *et al.*, 2015; Saathoff *et al.*, 2018). Alternatively, ExoI may act independently of SbcCD to suppress the aberrant recombinational pathway. Early studies suggested a strong physical interaction between Exo I exonuclease and RecA (Bedale *et al.*, 1991; Bedale *et al.*, 1993; Kowalczykowski *et al.*, 1994). Association of this 3′ exonuclease with RecA would be expected to strongly degrade recombinagenic 3′ ends preventing RecA from initiating recombination at these sites.

In an alternative interpretation of RecBCD function, a recent study and review speculated that chromosome cleavage may frequently occur during septation at cell division, resulting in double-strand breaks that require RecBCD for repair (Sinha *et al.*, 2017; Michel *et al.*, 2018). The authors based this argument primarily on the observation that the extensive degradation in *recBC* mutants centers upon the *dif* locus, where chromosomes ultimately separate as cells divide. However, several observations argue against this possibility. If the defects in *recBC* mutants were due to the presence of double-strand breaks, then *recA* mutants, should be similarly, or more severely affected. Yet, in their initial study, the authors failed to examine or address *recA* mutants (Sinha *et al.*, 2017). However, *recA* mutants grow at rates similar to wildtype cultures, and, unlike *recBC* mutants, maintain this region of the chromosome normally (Courcelle *et al.*, 2015; Wendel *et al.*, 2018). Further, these authors and previous investigators all note that DNA breaks are not detected on the chromosome of *recA* mutants (Pennington and Rosenberg, 2007; Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015; Sinha *et al.*, 2018). To explain the absence of DNA breaks in *recA* mutants, a subsequent study from this group proposed that the septation-induced breaks in *recA* genomes are degraded and therefore undetectable (Sinha *et al.*, 2018). However, based on the viability of *recBC* mutants, ~90% of cells in culture would be experiencing these septation-induced breaks. Synthesis and subsequent degradation of genomes at this frequency would be expected to slow culture growth considerably and generate degradation intermediates that should be easily detectable, neither of which are observed (Wendel *et al.*, 2014; 2018; Courcelle *et al.*, 2015; Sinha *et al.*, 2018). Models proposing frequent double-strand breaks also fail to address how the inactivation of exonucleases SbcCD and ExoI, which are not essential for double-strand break repair, would restore *recBCD* mutant growth defects, or why maintaining the terminus region of the chromosome in the absence of exonucleases would depend on RecA (Templin *et al.*, 1972; Lloyd and Buckman, 1985; Wendel *et al.*, 2018). Finally, as shown here, plasmids containing two replisomes require RecBC to propagate but do not require RecA and are actually stabilized in RecA's absence. These plasmids lack *dif* sequences, and segregate prior to and independent from cell division, and would therefore not be subject to septation-induced breaks. These observations are all inconsistent with the idea that the requirement for RecBCD is due to double-strand breaks caused by cell division, and argue strongly for its role in joining DNA ends of convergent forks independently of recombination or RecA.

It is also worth considering that although current models for RecBCD in double-strand break repair propose that RecBCD acts prior to RecA, the early *in vivo* studies led several independent labs to conclude that RecBCD acted after RecA, at a late step in completing the recombination reaction (Wilkins, 1969; Hall and Howard-Flanders, 1972; Birge and Low, 1974; Willetts, 1975). Current models placing RecBCD as an initiator are heavily derived from biochemical studies in which linear double-stranded substrates were used to characterize enzyme binding, helicase and exonuclease activities (reviewed in Dillingham and Kowalczykowski 2008; Yeeles and Dillingham 2010; Smith, 2012). The

initial concept that RecBC acts late in recombination arose from the observation that although *recA* mutants receiving an F′ factor were unable to transfer chromosomal genes to another cell, recBC mutants could do so at frequencies that approached those of wild-type cells. However, over time (~1 generation), this ability rapidly declined (Wilkins, 1969; Hall and Howard-Flanders, 1972; Willetts, 1975). The authors inferred that recombination proceeds beyond the point at which the incoming DNA is joined to the chromosome in *recBC* mutants, but that *recA* mutants are blocked prior to this event. In recombinational crosses between Hfr and F⁻ strains carrying noncomplementing mutations in *lacZ*, Birge and Low found that although *recA* mutants were entirely blocked, *recBC* mutants initially produced beta-galactosidase within twofold of those seen in wildtype cells, indicating that recombination reactions progressed beyond the point where transcribable, mutation-free copies of LacZ+ were produced (Birge and Low, 1974). However, although these recombination intermediates were detectable, the completion of these recombination events was impaired in the absence of *recBC* and viable LacZ+ recombinant progeny were reduced 100–1000-fold. The authors inferred that "early steps in recombination can proceed efficiently in RecB- and RecC-strains, but that late steps, such as the degradation of excess DNA 'tails,' might be defective". Using combinations of single and double mutants, Willetts confirmed these previous studies and suggested that recombination proceeded past the first joining reaction of the two DNA molecules, but that RecBC was required for a second joining needed "to generate a circular unit" that could be inherited (Willetts, 1975). These interpretations are strikingly consistent with RecBCD's apparent role in completing replication on the chromosome, and may suggest that RecBCD function during recombinational events is similar to its role in completing replication (Courcelle *et al.*, 2015).

Experimental procedures

Strains and plasmids

All strains used in this work are derived from SR108, a *thyA deoC* derivative of W3110 (Mellon and Hanawalt, 1989) and listed in Table 1. pBR322 contains a ColE1 origin of replication (Bolivar *et al.*, 1977). pCL01 is a derivative of pCB104 (Boyd and Sherratt, 1995) containing an ampicillin-resistance cassette from pBR322. Plasmid constructions were performed according to published protocols for in vivo recombineering (Yu *et al.*, 2000; Sawitzke *et al.*, 2012). Briefly, primers 5′gtcggttcagggcagggtcgtggatccactttagttacaacatacttattcgcggaacccctatttgttt and 5′ggcggtttgcgtattgggcgcatattagttacaacatcctatatggtctgacagttaccaatgc were used to amplify the *amp*^{R} gene from pBR322. 0.2 µg gel purified PCR product was then combined with 0.5 µg BamHI-digested pCB104 and amplified for 25 cycles using Pfu Turbo Polymerase (Agilent). PCR products were examined by agarose gel electrophoresis and products running larger than 5kb were gel purified and transformed into recombineering strain DY329 (Yu *et al.*, 2000) to generate the ampicillin resistant plasmid. A *chi* sequence was then removed from the plasmid using primer sets

Table 1. Strains and plasmids used in this study

Strain/Plasmid	Relevant genotype	Reference/ Construction
SR108 parental	λ⁻ *thyA deo IN(rrnD-rrnE)*	Mellon and Hanawalt (1989)
HL922	SR108 *recB21C22 argA81::*Tn*10*	Courcelle *et al.* (1997)
CL851	SR108 *recB21C22 argA81::*Tn*10 recA::cam*	Chow and Courcelle (2007)
HL923	SR108 *recD1011 argA81::*Tn*10*	Courcelle *et al.* (1997)
CL726	SR108 *recD1011 argA81::*Tn*10 recA::cam*	Chow and Courcelle (2007)
CL542	SR108 *recA::cam*	Chow and Courcelle (2007)
CL039	SR108 *xonA::cam*	Courcelle and Hanawalt (1999)
CL718	SR108 *xonA::Cat300 D(srlR-recA)306::*Tn*10*	Wendel *et al.* (2018)
CL2344	SR108 *sbcCD::Gm*	Wendel *et al.* (2018)
CL3535	SR108 *sbcCD::Gm D(srlR-recA)306::*Tn*10*	Wendel *et al.* (2018)
CL2357	SR108 *xonA::Cat300 sbcCD::Gm*	Wendel *et al.* (2014)
CL3539	SR108 *xonA::Cat300 sbcCD::Gm D(srlR-recA)306::*Tn*10*	Wendel *et al.* (2018)
CL2542	SR108 *xonA::Cat300 sbcCD::Gm recB21C22 argA81::*Tn*10*	Wendel *et al.* (2018)
CL2575	SR108 *xonA::Cat300 sbcCD::Gm recB21C22 argA81 D(srlR-recA)306::*Tn*10*	Wendel *et al.* (2018)
CL2456	SR108 *recG6200::tet857*	P1 transduction of *recG6200::tet857* from TP538 (Murphy *et al.*, 2000) into SR108
CL2579	SR108 *recG6200::tet857 recA::cam*	P1 transduction of *recA::cam* from CL542 (Courcelle *et al.*, 1997) into CL2456

5′attgctgataaatctgga/5′ctttggaatccagtccctcttcctcctgctgatctg-cgacttatcaac and 5′tccagatttatcagcaat/5′gttgataagtcgcagat-cagcaggaggaagagggactggattccaaag were used to amplify overlapping fragments of the plasmid template using Pfu Turbo Polymerase (Agilent). The fragments were then joined and transformed using Gibson assembly (New England Biolabs) to generate pCL01.

Growth rates

Wells containing 0.1-ml cultures were grown in LB medium supplemented with 10μg ml^{-1} thymine (LBthy) at 37°C with agitation in a 96-well microtiter dish. Absorbance at 630 nm was measured over time using a BIO-Whittaker ELx808 plate reader. The number of viable colonies per ml in each culture was determined at the start of every experiment. Equal numbers of viable cells were compared in each case (Courcelle *et al.*, 2012).

Transformation efficiency

Electro-competent cells were prepared by growing a 100-fold dilution of a fresh overnight culture in 10 ml LBthy to an OD600 of 0.4. Cells were then pelleted, and serially washed with 30 ml water, 30 ml 10% glycerol and then resuspended in 200 μl of 10% glycerol and stored at −80°C. To determine transformation efficiency, 40 μl of competent cells were mixed with 50ng of plasmid and electroporated at 1.8 kV with capacitance of 25 μFD and resistance of 200 Ohms and allowed to recover at 37°C for 30 min in 1 ml SOC media. The transformation reactions were then diluted and aliquots were spread on LBthy plates with and without 50 μg ml^{-1} ampicillin to determine the number of transformants and viable cells respectively. Colonies were counted following overnight incubation at 37°C. The same preparations of competent cells were used for all strains when comparing pBR322 and pCL01. The relative transformation efficiency of each strain was calculated as the ratio of transformants per viable cells in the mutant cultures to the transformants per viable cells in wildtype cultures.

Plasmid stability

Cells from overnight cultures of strains containing the plasmid grown in LBthy medium with 50 μg ml^{-1} ampicillin were pelleted and used to inoculate 10 ml cultures of LBthy medium at 1:1000 dilution. Cultures were grown without ampicillin selection at 37°C with aeration overnight. The resulting cultures were then sampled to determine the ratio of cells retaining the plasmid and used to reinoculate 10 ml LBthy medium at 1:1000 dilution. This was repeated for three iterations or a total of 30 generations. To determine plasmid retention, 10-μl aliquots of serial 10-fold dilutions were spotted on LBthy plates in the presence or absence of 50 μg ml^{-1} ampicillin. Colonies were counted following overnight incubation at 37°C and compared to establish the percent of plasmid-containing cells (Wendel *et al.*, 2014).

Southern analysis of plasmid forms

To purify total genomic DNA, 0.75 ml aliquots of culture containing the plasmid grown in LBthy medium with 100 μg ml^{-1} ampicillin were placed into 0.75 ml ice cold 2 × NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA). Each sample was pelleted, resuspended in 140 μl of 1 mg ml^{-1} lysozyme and 0.2 mg ml^{-1} RNaseA in TE (10 mM Tris, pH 8.0, 1 mM EDTA), and lysed at 37°C for 30 min. Proteinase K (10 μl, 10 mg ml^{-1}) and Sarkosyl [10 μl, 20% (wt/wt)] were then added and incubation continued for 30 min. Samples were then serially extracted with 4 volumes phenol/chloroform (1/1) and 4 volumes chloroform followed by dialysis for 1 h on 47 mm Whatman 0.05-μm pore disks (Whatman #VMWP04700) floating on a 250-ml beaker of TE (1 mM Tris, pH 8.0, 1 mM EDTA).

Total genomic DNA from each strain was then partially digested with SacII (for strains containing pBR322) or ApaI (for strains containing pCL01) (Thermo Fisher Scientific). Restriction sites for these enzymes are absent on each plasmid. Samples were then extracted with 1 volume of chloroform. Equal cell equivalents were loaded on a 0.5% or 1.0% agarose gel as indicated, and electrophoresed in 1 × TBE (220 mM Tris, 180 mM Borate, 5 mM EDTA, pH 8.3). Gels were transferred to Hybond N+ nylon membranes (Amersham-GE Healthcare) and probed with either ^{32}P-labeled pBR322 or pCL01. Probes were prepared by nick translation according to the protocol supplied by Roche using [α-^{32}P]dCTP (PerkinElmer). Radioactivity was visualized using a Storm 840 and its associated ImageQuant Software (Molecular Dynamics) (Wendel *et al.*, 2014).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article

Supplementary Information

RecBCD, SbcCD and ExoI process a substrate created by convergent replisomes to complete DNA replication

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Running title: SbcCD ExoI and RecBCD complete bidirectional plasmid replication

Contains Supplemental Figures S1, S2, S3, and S4.

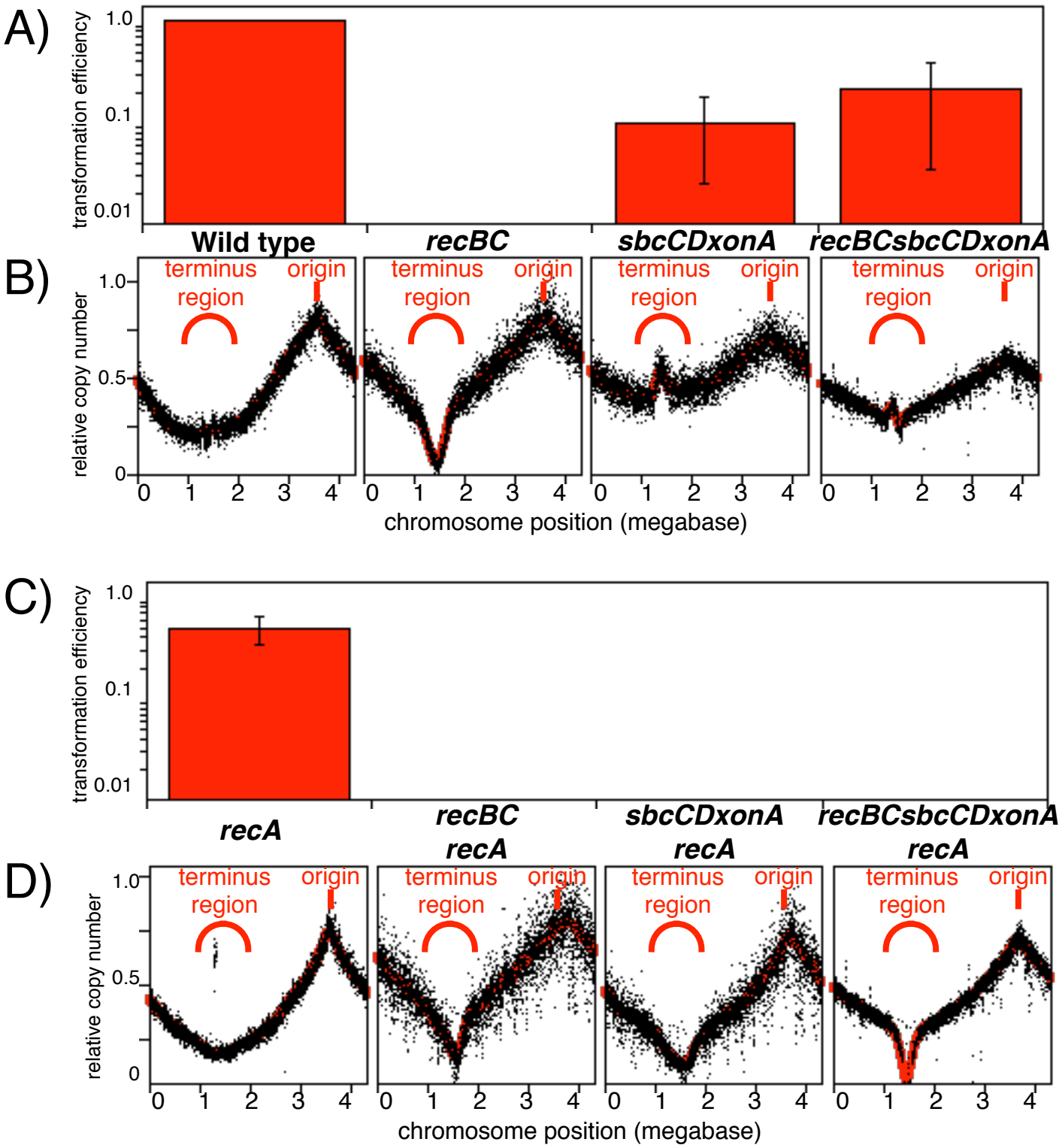

Figure S1. The ability to maintain the chromosome region where replication completes, correlates with the ability to maintain two-replisome plasmids. A&C) The transformation efficiency of the two-replisome plasmid, relative to wild type cells, is shown for the strains indicated. Error bars represent the standard error of at least two independent experiments. Data from Fig 2. B&D) To profile the replication pattern in each strain, genomic DNA from replicating cultures was purified, fragmented, and sequencing using high-throughput sequencing. Sequence read frequencies at each chromosome loci, normalized to stationary phase cells, are plotted. An 8kb floating average of sequence frequency is plotted in red. Data from (Wendel et al., 2014; Wendel et al., 2018).

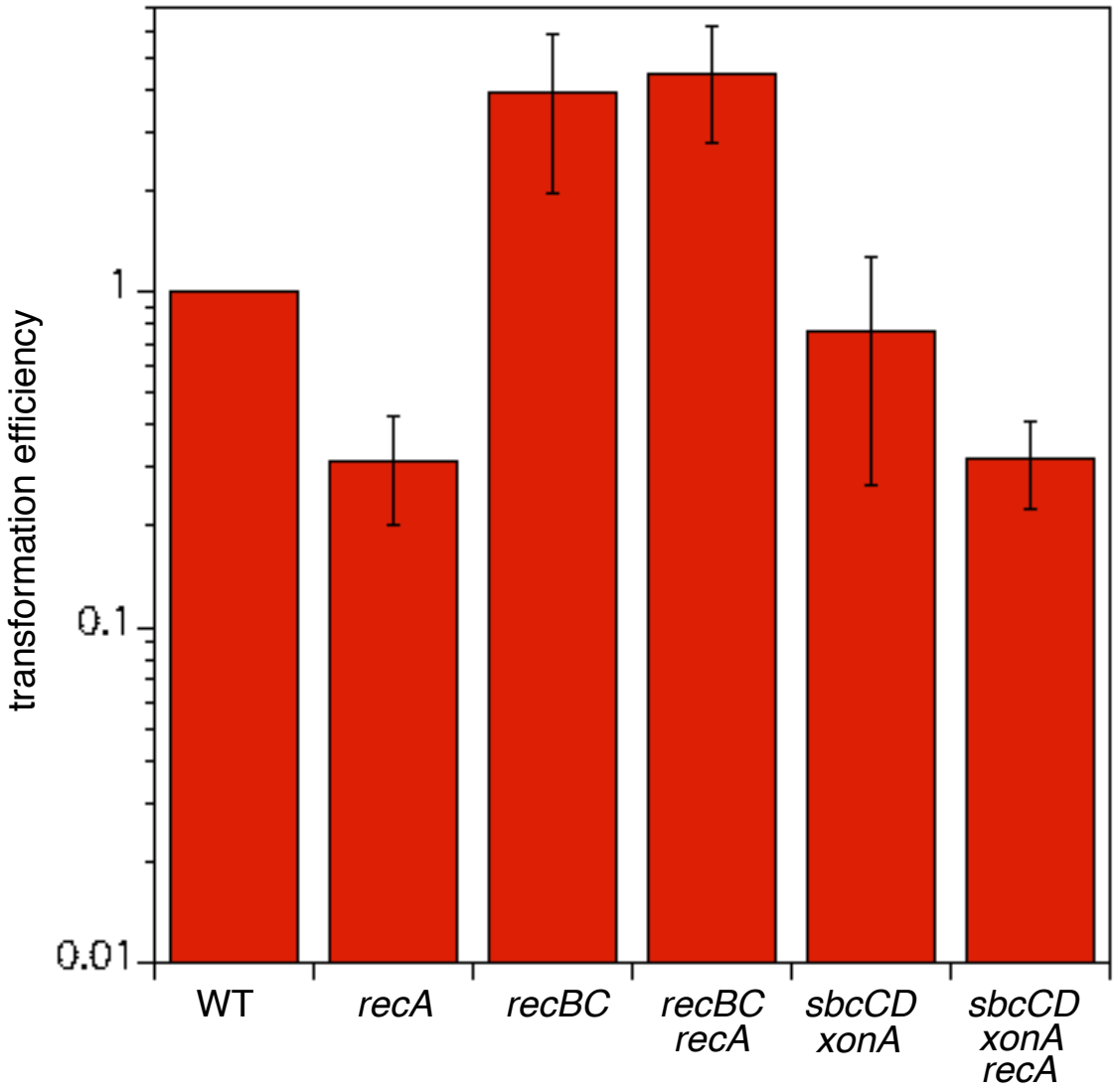

Figure S2. Transformation of the one replisome plasmid, pREP4 does not depend on the enzymes required to complete replication on the chromsome. The transformation efficiency, relative to wild type cells, is shown for the strains indicated following electroporation of competent cells with 50ng of pREP4. pREP4 utilizes a p15 unidirectional origin of replication that is distinct from that of the ColE1 origin used by pBR322. Error bars represent the standard error of two independent experiments.

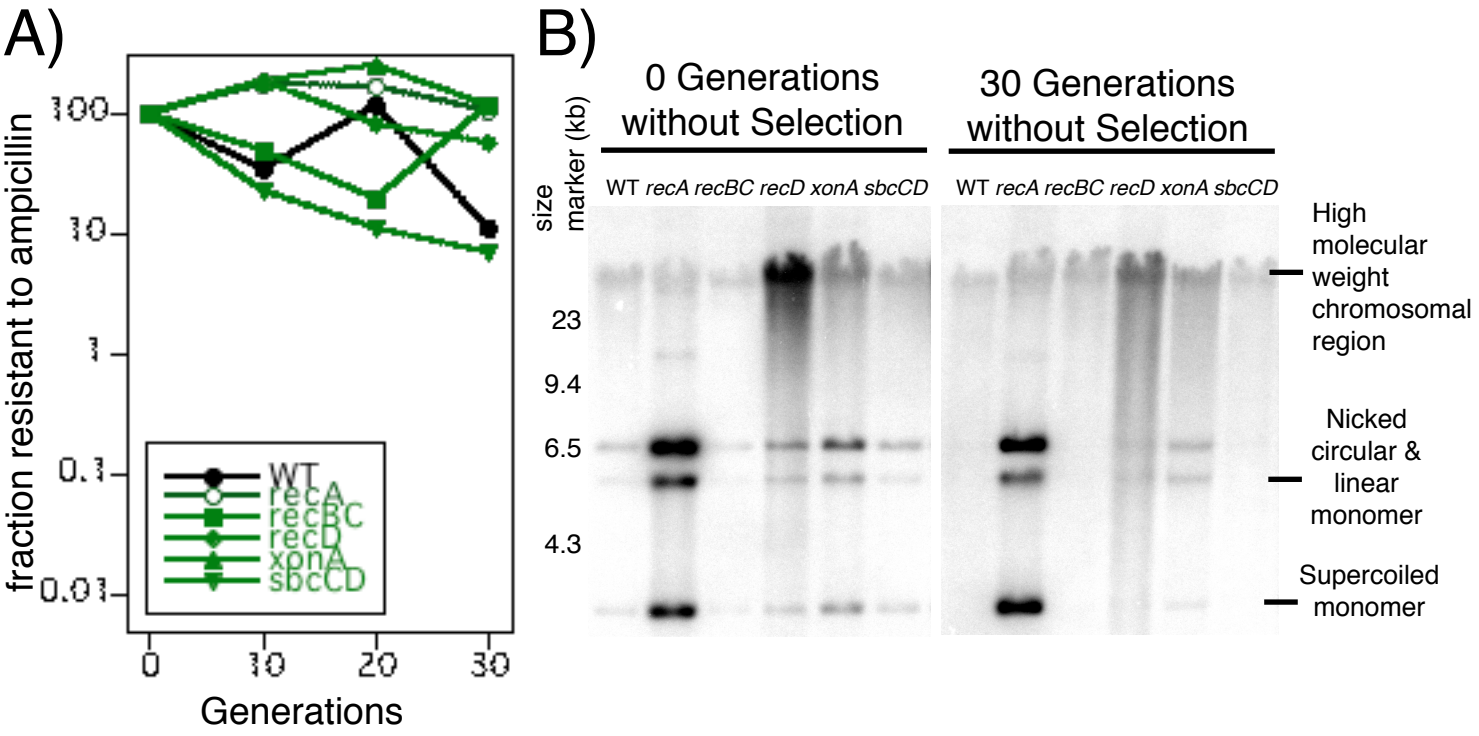

Figure S3. Plasmids containing the *E. coli* origin of replication, *oriC*, integrate into the chromosome at high frequency due to the extensive homology shared between the chromsome and plasmid DNA (Yasuda and Hirota, 1977; Messer et al., 1978; Meijer et al., 1979)**. These integration events prevent a direct analysis of stability when using this plasmid.** A) *Cultures containing the two-replisome plasmid, pOriC-2, maintain resistence to ampicillin, even in the absence of selection*. pOriC-2 contain the E.coli origin of replication and a gene conferring resistence to ampicillin (Sawitzke et al., 2012). Cultures containing pOriC-2 were grown without selection. Serial dilutions of the culture were then plated with and without ampicillin selection to determine the fraction of cells that remained resistent to the antibiotic selection marker. B) *Although cultures remain resistant to ampicillin, the plasmid is not maintained as a minicircle, and frequently integrates into the chromsomal DNA*. Total genomic DNA from cells containing the one-replisome or two-replisome plasmid was purified and analyzed by southern analysis following agarose gel electrophoresis using P32-labeled pOriC-2 as a probe.

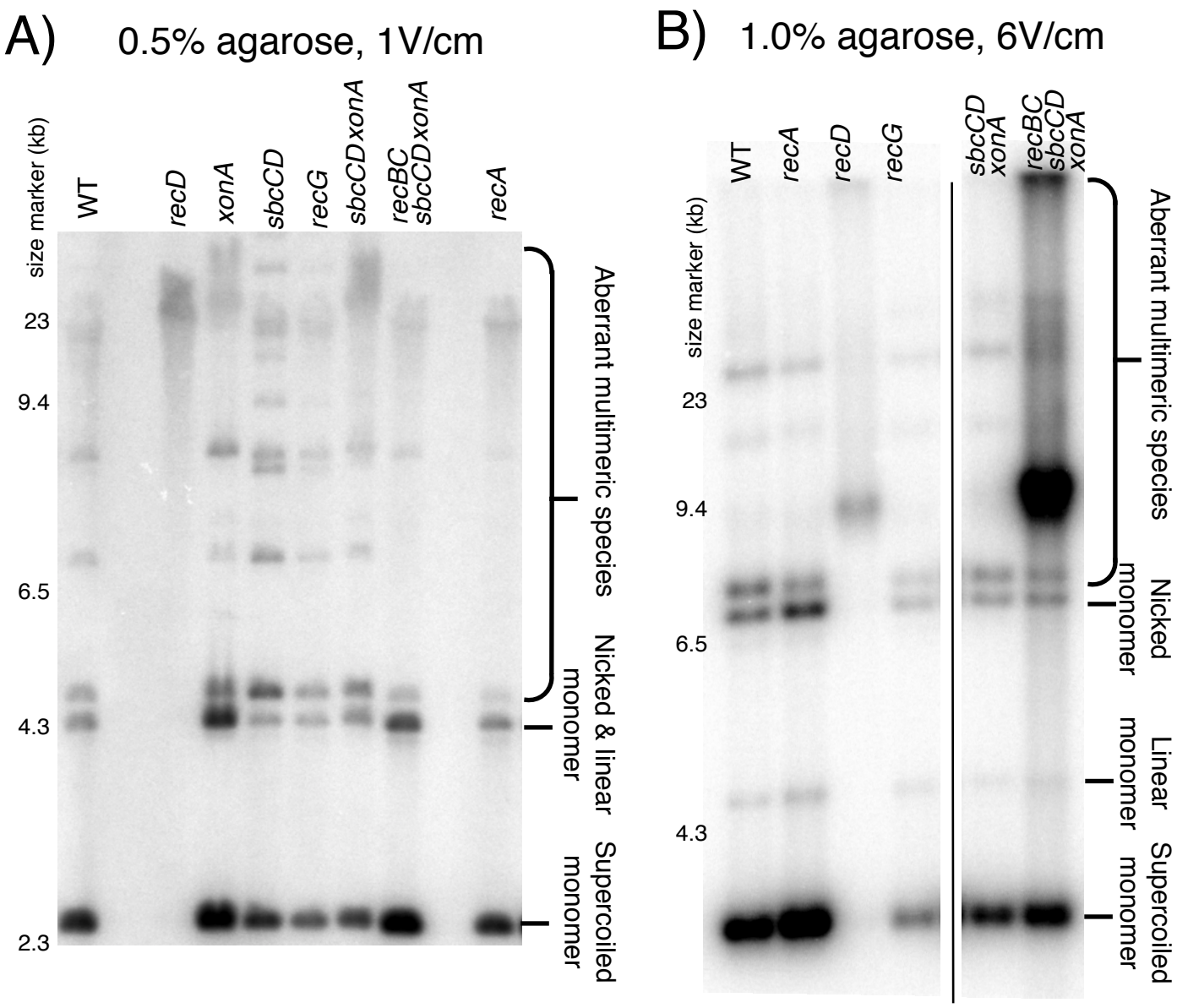

Figure S4. Other examples of the two-replisome plasmid pCL01 in various strains showing the variability observed in backgrounds where the plasmid is unstable. A&B) *Representative Southern analyses of plasmid pCL01 grown in the indicated strain is shown*. Total genomic DNA from cells containing the two-replisome plasmid was purified and analyzed by southern analysis following agarose gel electrophoresis using P32-labeled pCL01 as a probe. In (A) DNA was electrophoresed in 0.5% agarose at 1 V/cm. Under these conditions, the DNA is resolved primarily based upon size, and is useful for resolving the different higher order molecular weight species. In (B) DNA was electrophoresed in 1.0% agarose at 6 V/cm. Under these conditions, the DNA shape plays a larger factor in the migration rate of the DNA. These conditions allow us to resolve the broken linear monomeric species from the nicked circular monomers, but often fails to resolve differences between higher order molecular weight species. As is clear, none of the mutants examined accumulate linear monomeric DNA to an extent above that seen in wild type cells.