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Replication fork inhibition in *seqA* mutants of *E. coli* triggers replication fork breakage

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Abstract

SeqA protein negatively regulates replication initiation in *E. coli* and is also proposed to organize maturation and segregation of the newly-replicated DNA. The *seqA* mutants suffer from chromosomal fragmentation; since this fragmentation is attributed to defective segregation or nucleoid compaction, two-ended breaks are expected. Instead, we show that, in SeqA's absence, chromosomes mostly suffer one-ended DNA breaks, indicating disintegration of replication forks. We further show that replication forks are unexpectedly slow in *seqA* mutants. Quantitative kinetics of origin and terminus replication from aligned chromosomes not only confirm origin overinitiation in *seqA* mutants, but also reveal terminus underreplication, indicating inhibition of replication forks. Pre/post-labeling studies of the chromosomal fragmentation in *seqA* mutants suggest events involving single forks, rather than pairs of forks from consecutive rounds rear-ending into each other. We suggest that, in the absence of SeqA, the sister-chromatid cohesion "safety spacer" is destabilized and completely disappears if the replication fork is inhibited, leading to segregation fork running into the inhibited replication fork and snapping it at single-stranded DNA regions.

Keywords

seqA; *recA*; *recBC*; *ruvABC*; ori/ter ratio; chromosomal fragmentation

Introduction

Double-strand DNA breaks fragment chromosomes and are the most dangerous DNA lesions, as well as, perhaps, the most complicated ones to repair. Chromosomal fragmentation is invariably lethal unless the double-strand breaks are mended (Bonura & Smith, 1975, Dahm-Daphi *et al.*, 2000, Freifelder, 1965, Iliakis, 1991, Kouzminova & Kuzminov, 2012, Resnick, 1976). In unicellular organisms, fragmented chromosomes are repaired via homologous recombination, catalyzed in bacteria by the RecBCD (or its analogs), RecA and RuvABC enzymes (Dillingham & Kowalczykowski, 2008, Kuzminov, 2011, Roca & Cox, 1997, West, 1997). Yet, even the restoration of physical DNA continuity does not guarantee that the chromosome returns to a functional state, as double-strand break

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misrepair causes chromosomal rearrangements or dysfunctional chromosomes (Olive, 1998, Rothkamm & Löbrich, 2002, Vamvakas *et al.*, 1997).

Because of the enzymatic complexity at the DNA level and of the logistic complexity at the chromosomal level (to prevent misrepair), it would be more efficient for the cell to avoid repair by recombination altogether. Indeed, cells prevent most of the potential double-strand breaks using various chromosomal-fragmentation-avoidance functions. When one of these avoidance functions is mutated in *E. coli*, the otherwise viable *recA* mutants become inviable (co-lethal) with this mutation, because now they can neither avoid double-strand breaks, nor repair them. In order to reveal pathways to avoid spontaneous chromosomal fragmentation, we have previously isolated mutants co-lethal with the *recA* defect (Kouzminova *et al.*, 2004). One such chromosome-fragmenting mutant inactivated the *seqA* gene for the negative regulator of initiation of the chromosomal replication (Lu *et al.*, 1994, von Freiesleben *et al.*, 1994). Inactivation of SeqA increases the number of replication origins per cell, suggesting overinitiation (Boye *et al.*, 1996, Riber *et al.*, 2006, von Freiesleben et al., 1994).

In an attempt to understand the nature of double-strand DNA breaks in *seqA* mutants, we considered the in vitro properties of the SeqA protein. The 20 kDa SeqA protein binds close pairs of hemimethylated GATC sites positioned on the same side of DNA duplex (Brendler *et al.*, 2000). The *oriC* sequence has several such pairs, explaining SeqA binding and its role in the regulation of *oriC* firing (Waldminghaus & Skarstad, 2009), but appropriately-spaced pairs are also found all over the chromosome, on average once every 2.5 kbp (Brendler et al., 2000), suggesting that SeqA binding is not limited to the origin. The number of SeqA molecules per cell, around 1,000 (Slater *et al.*, 1995), also suggests SeqA binding beyond the replication origin. Judging by in vitro behavior of its self-association domain, SeqA protein is capable of forming spiral filaments that, via SeqA's DNA-binding domain, should be able to bind hemimethylated GATC sites in the nascent duplexes behind replicaton forks (Brendler et al., 2000). In vivo, SeqA forms foci, whose number equals the number of replication forks (Brendler et al., 2000) or, in rapidly-growing cells, the number of replication bubbles (pairs of forks) (Molina & Skarstad, 2004, Morigen *et al.*, 2009). Finally, ChIP-on-chip studies find SeqA mostly behind the progressing replication forks, with an additional minor binding at the replication origin (Waldminghaus *et al.*, 2012). All these observations are encompassed by the current model assigning SeqA a broad role in organizing the newly-replicated DNA in *E. coli*, as well as in regulating origin firing (Kuzminov, 2013, Sawitzke & Austin, 2001, Waldminghaus & Skarstad, 2009). Yet, consideration of the known or suspected SeqA activities fails to clarify the nature of chromosomal fragmentation in *seqA* mutants.

Maybe the phenotypes of *seqA* mutants will shed light on how SeqA acts to avoid chromosomal fragmentation? As already mentioned, *seqA* mutants are reported to have an increased number of replication origins per cell (Boye et al., 1996, Riber et al., 2006, von Freiesleben et al., 1994), yet normal (Camara *et al.*, 2005) or somewhat elevated (Bach & Skarstad, 2004, Riber et al., 2006) ori/ter ratios, suggesting mostly normal replication, but problems with subsequent *segregation* of complete chromosomes. SeqA was proposed to aid segregation of daughter DNA duplexes by channeling newly-replicated DNA to the places

of new nucleoid condensation (Sawitzke & Austin, 2001). The possibility of segregation problems in *seqA* mutants is supported by their partitioning-minus (*par*) phenotype (von Freiesleben *et al.*, 2000, Weitao *et al.*, 1999). If caused by segregation problems, the double-strand DNA breaks in *seqA* mutants may ultimately form during septation, for example due to guillotining of incompletely-segregated nucleoids. Finally, the *seqA* mutants have increased supercoiling (Weitao *et al.*, 2000), which could potentially exacerbate segregation problems or lead to DNA compaction-induced double-strand breaks. Again, the nature of chromosomal fragmentation in *seqA* mutants remains unclear.

From the above discussion, there are four distinct chromosome transactions at which SeqA, as the protein associated with nascent DNA, may act at to avoid chromosome fragmentation (Fig. 1). The prokaryotic chromosome cycle is dramatically different from the eukaryotic one in that all the major transitions (decompaction – replication – sister chromatid cohesion – segregation – recompaction) happen one after another, concurrently, in a sliding window migrating from the replication origin to the chromosome terminus (Kuzminov, 2013, Kuzminov, 2014) (Fig. 1). First, SeqA hyperstructure may act to support replication forks (Norris *et al.*, 2000), preventing replication fork disintegration. Second, by complexing nascent DNA and prolonging sister-chromatid cohesion (SCC) (Joshi *et al.*, 2013), SeqA may organize a “safety spacer” (Fig. 1) to prevent breakage at single-strand regions, like the one proposed to happen during ligase-deficient replication (Kouzminova & Kuzminov, 2012). Third, SeqA may protect sister DNA duplexes during segregation, by providing a conduit to channel them along, as proposed (Sawitzke & Austin, 2001). Fourth, SeqA may organize proper recompaction, suggested by the tighter nucleoids in *seqA* mutants (Weitao et al., 1999). In order to distinguish between the four possibilities (Fig. 1), we have investigated the replication complexity and chromosome dynamics, as well as the format of chromosomal fragmentation, in *seqA*-deficient conditions.

Results

The Δ*seqA* mutants are induced for SOS and depend on double-strand break repair

We have previously reported (Kouzminova et al., 2004, Rotman *et al.*, 2009) that the *seqA* mutants are dependent on RecA for viability, indicating chromosomal problems that require recombinational repair. Chromosomal damage is defined as DNA damage that blocks the chromosome cycle (Fig. 1) (Kuzminov, 2013); in bacteria, chromosomal damage is mended by homologous recombination, of which RecA is the central activity (Kuzminov, 1999, Kuzminov, 2011). There are two pathways within the recombinational repair in *E. coli*: one is the RecBC- and RuvABC-dependent pathway for repair of double-strand DNA breaks, the other is the RecFOR-dependent pathway for repair of persistent single-strand gaps (Kuzminov, 1999). The single Δ*seqA* mutant is cold-sensitive, growing similarly to wild type at 45°C, but showing a significant lag as the temperature decreases, as was observed before (Lu et al., 1994, Rotman et al., 2009). A double Δ*seqA* Δ*recF* mutant grew identically to the single Δ*seqA* mutant (Fig. 2A), indicating no requirements for persistent single strand gap repair. At the same time, the Δ*seqA* allele in combination with Δ*recA*, Δ*recBCD* and Δ*ruvABC*, all parts of the double strand break repair, was barely alive at 45°C and could not form colonies at lower temperatures (Fig. 2A), indicating formation of spontaneous double-

strand DNA breaks in *seqA* mutants. In other backgrounds, such as MG1655 or DH5α *recA* +, the double Δ*seqA recA* mutants managed to form colonies, but Δ*seqA recBCD* and Δ*seqA ruv* combinations were still lethal (for example, Fig. S1). This, and the fact that synthetic lethals in combination with *seqA* are readily suppressed (Rotman et al., 2009), are likely behind the previous reports that *seqA recA* double mutants are viable (Lu et al., 1994, Sutera & Lovett, 2006, Weitao et al., 2000).

Chromosomal fragmentation in *seqA* mutants should induce the SOS response, a general transcriptional response of bacterial cells to chromosomal lesions (Kuzminov, 1999, Little & Mount, 1982). Indeed, the levels of SOS induction in three different *seqA* alleles (Fig. S2): *seqA-4*, interrupted by pRL27 (Kouzminova et al., 2004), Δ*seqA::tet* (Lu et al., 1994) and Δ*seqA20::kan* are all significantly increased over the background (Fig. 2B), confirming our previous reports (Kouzminova et al., 2004, Rotman & Kuzminov, 2007). The induction was higher at lower temperatures, but the background was elevated, too, making the absolute increase similar at both temperatures (Fig. 2B). The SOS induction in Δ*seqA* mutants was not due to the disruption of the downstream *pgm* gene (Fig. 2B). We conclude that the RecA-dependence of *seqA* mutants is indeed due to the ongoing chromosomal damage.

The Δ*seqA* mutants fragment their chromosome

Dependence on the genes of the double-strand break repair in combination with SOS induction indicates chromosomal fragmentation (Bradshaw & Kuzminov, 2003, Kouzminova & Kuzminov, 2004, Kouzminova et al., 2004), detected in bacterial circular chromosomes as genomic DNA smearing out of the wells in pulsed-field gels (Michel *et al.*, 1997). To reveal chromosomal fragmentation, *recBC*-deficient conditions are used to prevent both the repair and degradation of the fragmented chromosomes (Khan & Kuzminov, 2012, Kouzminova & Kuzminov, 2006, Michel et al., 1997). When we propagated Δ*seqA* mutants in *recBC*-deficient conditions, by either inactivating the temperature-sensitive RecBC protein at the non-permissive temperature (Rotman & Kuzminov, 2007) or by expressing the RecBCD-inhibiting protein Gam of phage lambda (Friedman & Hays, 1986), and detected DNA in the resulting pulsed-field gel by ethidium bromide-staining, we observed significant fragmentation in both RecBCD+ (Fig. S3, lines 8, 11 and 12) and *recBCD* mutant (Fig. S3, lines 9 and 10) conditions.

However, when measured quantitatively by labeling chromosomal DNA with ^{32}P-orthophosphate, chromosomal fragmentation in the single Δ*seqA* mutants is not much different from the one in wild type cells, or *recBC*(Ts) mutants, whereas fragmentation in the Δ*seqA recBC* cells is still significant (Fig. 2CD), as was reported before (Kouzminova et al., 2004, Rotman et al., 2009, Rotman & Kuzminov, 2007). After fragmentation levels in the single mutants are taken into account, the double mutant fragmentation is 8.5% (Fig. 2D legend). A likely reason for the apparent fragmentation in the single Δ*seqA* mutant in the ethidium bromide-stained gels (Fig. S3) is the ~2X-higher DNA content of the *seqA* mutant cells ((Bach & Skarstad, 2004, Camara et al., 2005, von Freiesleben et al., 2000, von Freiesleben et al., 1994) and see below). Since the same amount of cells is collected during preparation of agarose plugs for PFGE, this leads to a significant overestimation of fragmentation in readouts with one order of magnitude linearity, like fluorescence of DNA-

bound ethidium bromide (Simmons *et al*., 2004). We conclude that not only do *seqA* mutant cells fragment their chromosome, but also that this fragmentation is all repairable in Rec+ cells, making *seqA* mutants dependent on the recombinational repair of double-strand breaks.

Replication fork disintegration in Δ*seqA* mutants

Next we asked at what stage of the chromosome cycle transition (Fig. 1) do the double-strand breaks happen in *seqA* mutants. Since these breaks are fully repairable by homologous recombination, they cannot happen before replication — otherwise they would have been irreparable, as there is no intact sister duplex to go to in unreplicated DNA (Fig. 3A, decompaction). Repairable double-strand DNA breaks happen during replication, or segregation or recompaction (Fig. 3A). The *seqA* mutants would be expected to fragment chromosomes during faulty segregation, consistent with the *par* phenotype of the *seqA* mutants (von Freiesleben et al., 2000, Weitao et al., 1999). The “segregation” breaks must be two-ended (Fig. 3A) and could result, for example, from chromosomal loop guillotining by the closing septum in the absence of hypothesized channeling of the newly-replicated DNA towards the places of formation of the new nucleoids (Brendler et al., 2000, Sawitzke & Austin, 2001). An alternative model, also predicting two-ended double-strand breaks, was their formation during re-compaction, for example because *seqA* mutants have tighter nucleoids (Weitao et al., 1999) due to increased negative DNA supercoiling (Weitao et al., 2000) (Fig. 3A). At the same time, the reported normal (Camara et al., 2005) or somewhat elevated (Bach & Skarstad, 2004, Riber et al., 2006) ori/ter ratios indicated no or mild replication problems in *seqA* mutants, making replication-dependent fragmentation the least likely model. Yet, in contrast to the other three possibilities, replication fork disintegration events would generate *one-ended* breaks, yielding a testable distinction about the presence of the terminus sequence on the chromosomal fragments (Kouzminova & Kuzminov, 2006) (Fig. 3A). Specifically, segregation-dependent and compaction-dependent two-ended double-strand breaks generate both the terminus-containing and the origin-containing chromosomal fragments, whereas replication fork disintegration always leaves the terminus on the circular (intact) part of the chromosome, generating only origin-containing chromosomal fragments (Fig. 3A).

We hybridized the fragmentation smear in the *seqA recBC*(Ts) cells with either origin-specific or terminus-specific probes (as we did before with the *dut* mutants (Kouzminova & Kuzminov, 2006)). If the fragmentation is due to two-ended double-strand breaks, both origin- and terminus-specific probes should reveal the same level of fragmentation, as shown by the sheared chromosomal DNA control (Fig. 3B, lanes 3 and 6). On the other hand, if the fragmentation is due to replication fork disintegration, then the terminus-specific probes should reveal no fragmentation, while the origin-specific probe should reveal even more fragmentation. We found that the terminus-specific probe reveals very little fragmentation in the Δ*seqA* mutants, while the origin-specific probe reveals double levels of fragmentation: 15.6% with the origin-specific probe (Fig. 3C) versus 8.5% in the overall chromosome (Fig. 2D). This finding rules out two-ended double-strand breaks and is therefore inconsistent with either segregation- or recompaction-induced double-strand DNA breaks in Δ*seqA* mutants. We conclude that the situation when all the subchromosomal

fragments in the *seqA* mutants carry replication origin, but no terminus, indicates the replication-dependent nature of double-strand DNA breaks in the absence of SeqA and raises the question about the nature of the replication defect in *seqA* mutants.

The Δ*seqA* defect elevates the ori/ter ratio, indicating slow replication forks

The conclusion about the replication defect in the *seqA* mutant was in disagreement with one particular chromosome metric, the ori/ter ratio, which is between 2 and 3 for rapidly-dividing *E. coli* cells with normal replication (Bremer & Dennis, 1996). Elevated to 4 and above, the ori/ter ratio means slower replication forks and signals replication defects (Fig. 4A, right) (Bird *et al.*, 1972, Kuong & Kuzminov, 2012, Lane & Denhardt, 1975). The reported normal (Camara et al., 2005) or somewhat elevated (Bach & Skarstad, 2004, Riber et al., 2006) ori/ter ratios suggested moderate or no replication defect in *seqA* mutants. Instead, the reports of increased number of origins in the *seqA* mutant cells (Lu et al., 1994, von Freiesleben et al., 1994), in combination with normal ori/ter ratios, suggested chromosome segregation problems (Fig. 4A, left), similar to the *obgE*-defective *E. coli* cells (Foti *et al.*, 2007, Kobayashi *et al.*, 2001). The segregation defect in the absence of SeqA would be consistent both with the theoretical analysis (Sawitzke & Austin, 2001) and the reported partitioning defect on *seqA* mutants (von Freiesleben et al., 2000, Weitao et al., 1999). Since our findings about chromosome fragmentation suggested a *replication* defect in *seqA* mutants, we reinvestigated the ori/ter ratio in the chromosome of *seqA* mutants.

The origin-to-terminus ratio was normalized to the wild type overnight cultures, in which it was close to 1:1. Growing cultures of wild type cells showed the expected 2:1 ratio of origin to terminus; in contrast, the Δ*seqA* mutants showed a two-fold increase in the ori/ter ratio (4:1) at 40°C and a three-fold increase (6:1) at 30°C (Fig. 4B and (Rotman et al., 2009)), close to the proposed limit of *E. coli*'s replication complexity (Zaritsky *et al.*, 2006). At the normal ori/ter = 2 there are two replication forks per chromosome, while there are 10 of them at the ori/ter = 6 (Fig. 4B inset). The Δ*seqA* mutants are sicker at lower temperatures, when ori/ter = 6, which indirectly supports the proposed replication complexity limit. The ori/ter ratios were lower in the original deletion-replacement *seqA* alleles (Δ*seqA19* and Δ*seqA-pgm* (Lu et al., 1994, Lu & Kleckner, 1994)), maybe because of the remaining 5′-part of the *seqA* gene. We conclude that the significantly increased ori/ter ratio in the Δ*seqA* mutants argues for a severe replication defect, rather than a partitioning defect (Fig. 4A). Specifically, replication forks must be slow in *seqA* mutants, like in the *rep* mutants, in which ori/ter ratio is similarly elevated in response to demonstrably slow replication forks (Lane & Denhardt, 1975).

Slowing down of replication forks may lead to accumulation of the extra replication bubbles in the origin part of the chromosome, detected as marker frequency profiles exponentially increasing towards the origin, as was reported for the *dut recBC*(Ts) mutants (Kouzminova & Kuzminov, 2008) and the *ligA*(Ts) Δ*recBCD* mutants (Kouzminova & Kuzminov, 2012) at the non-permissive temperatures. When replication forks are inhibited by hydroxyurea (Kuong & Kuzminov, 2012), or control over initiation is lost (Riber et al., 2006), a significant amount of additional replication bubbles accumulate in the limited *oriC*-centered chromosome segment. Although the marker frequency profile of a Δ*seqA* mutant looks

uniformly elevated relative to the WT profile, there is more elevation in the origin-centered 1 Mbp segment of the chromosome in the *seqA* mutant profile (Fig. 4C), indicating even slower replication forks and their additional accumulation around the origin.

The nature of the increased ori/ter ratio in Δ*seqA* mutants

There could be two dynamically-opposite scenarios leading to the deviation from the normal ori/ter = 2 ratio (Fig. 5A). High ori/ter ratio may develop in response to slow progress of replication forks, as cells with slow forks have to maintain more replication rounds per chromosome, to produce enough chromosomal DNA by cell division (Bird et al., 1972, Lane & Denhardt, 1975) (Fig. 5B). This situation could be distinguished from the wild type situation, by monitoring the kinetics of origin and terminus increase in the culture, as slower than expected populational increase of the terminus (Fig. 5D vs E). Alternatively, the same high steady-state ori/ter ratio may develop from more frequent replication initiations at the origin (Fig. 5C), which would force the cells to keep the normal rate of replication completion (Fig. 5D vs F), actively slowing down replication forks (otherwise the cells would suffocate from too much DNA). This situation differs from the wild type situation by faster than expected populational increase of the origin (Fig. 5D vs F).

To distinguish between the two possibilities, we determined the kinetics of the absolute increase in the origin versus terminus signals from aligned chromosomes in Δ*seqA* mutants relative to the wild type cells. The chromosomes were first aligned in the overnight cultures grown at 45°C, with ori/ter ratios close to 1 (Fig. 5A). The aligned cultures were then diluted into fresh medium and grown at 30°C, to exacerbate the *seqA* mutant phenotype. In WT cells, the kinetics of increase of the terminus became parallel to that of the origin after the *first* doubling, as expected (Fig. 5D vs G). In contrast, in the *seqA* mutant cultures, the rates of origin and terminus increase became the same after the *second* doubling (Fig. 5H). During the first doubling, origin in the *seqA* mutant replicated faster than origin in the wild type cells, after which the rate of origin increase became the same as in WT (Fig. 5H). The terminus did not increase during the first doubling in both WT and *seqA* mutant cells, as expected, but during the second doubling the terminus replicated slower in the *seqA* mutant (Fig. 5H). The rates of replication of both origin and terminus became the same in both WT and *seqA* mutant during the third doubling.

These differences in origin and terminus replication kinetics can be summarized by the ori/ter ratios. In the WT cells, the ori/ter ratio reached 2 during the first doubling and stayed essentially unchanged thereafter (Fig. 5I). In contrast, in the *seqA* mutant cultures, the ori/ter ratio increased from 1 to 3 during the first doubling (due to overinitiation) and from 3 to 5 during the second doubling (due to slow terminus replication), after which it stabilized (Fig. 5I). We conclude that the increased ori/ter ratio in the *seqA* mutants is a combination of initial replication overinitiation and subsequent inhibition of the replication fork progress.

Mechanisms of the replication fork disintegration in Δ*seqA* conditions

Could the inhibition of replication forks in the absence of SeqA directly cause replication fork disintegration in *seqA* mutants? Or could the replication fork disintegration be an indirect outcome of the “replication fork crowding”, since there are five times more

replication forks per chromosome in *seqA* mutants? The three established models of replication fork (RF) disintegration are: 1) RF collapse at single-strand interruptions in the template DNA (Hanawalt, 1966, Kuzminov, 1995b, Skalka, 1974) (Fig. 6A, center); 2) RF regress-split, according to which replication fork reversal turns it into a Holliday junction with its subsequent resolution (Kuzminov, 1995c, Michel, 2000, Morgan & Severini, 1990) (Fig. 6A, right); 3) RF rear-ending into a stalled replication fork of the previous round (Bidnenko *et al.*, 2002, Grigorian *et al.*, 2003, Nordman *et al.*, 2007, Simmons et al., 2004) (Fig. 6A, left). Additional models are possible (like breakage of inhibited replication forks (Kuzminov, 1995c) (Fig. 6A, top), awaiting their mechanistic formulation.

RF collapse at nicks in template DNA is consistent with the bulk of chromosomal fragmentation in the *dut* mutants of *E. coli*, where DNA-uracil incorporation followed by excision maintains an elevated steady-state level of strand breaks, detectable for example as increased relaxation (decrease of the supercoiled form) in the total plasmid DNA (Kouzminova & Kuzminov, 2006). However, it is unclear how the *seqA* defect would lead to elevated levels of strand breaks. In fact, in agreement with others (Weitao et al., 2000), we detected an *increase* of the supercoiled form in the total plasmid DNA in Δ*seqA* mutants relative to wild type cells (Fig. 6B), suggesting fewer strand breaks in the DNA of Δ*seqA* mutants and arguing against the RF collapse model.

The RF regress-split model could fit the situation in the *seqA* mutants better, because inhibited replication forks (for example, in the *rep* and *dnaB* mutants (Seigneur *et al.*, 2000) or in UV-irradiated cells (Khan & Kuzminov, 2012)) are prone to reversal and turning into Holliday junctions, that are then resolved by RuvABC, splitting the replication structure (Fig. 6A, right). However, the equally poor viability of *seqA recA*, *seqA recBCD* and *seqA ruv* mutants (Fig. 2A) argues against this model for *seqA* mutants. Indeed, a typical pattern of the RF regress-split situation is synthetic lethality with the *recBCD* defect only, and at the same time significantly milder effects of the *recA* and *ruv* defects (De Septenville *et al.*, 2012, Flores *et al.*, 2001, Seigneur *et al.*, 1998).

Basically, of the three established models of replication fork disintegration, only the RF rear-ending model was *a priori* consistent with the situation in *seqA* mutants, dovetailing with the replication fork crowding in the absence of SeqA (Fig. 4B inset). Indeed, different replication bubbles may run at different rates, explaining occasional rear-ending of a fast replication fork into a slower-moving replication fork of the previous replication round, with release of sub-chromosomal fragments (Fig. 6A, left). Note that, in contrast to all other models of replication fork disintegration that predict equal fragmentation of old and new DNA strands, RF rear-ending model predicts no fragmentation of “old” DNA strands, synthesized before the fragmentation events.

To test this prediction, we measured the degree to which chromosomal fragmentation in *seqA* mutants affects the “old” versus “new” DNA strands. By labeling cells in non-fragmenting conditions and removing the label from the growth medium before the switch to the fragmenting conditions, we specifically detect fragmentation of the old strands. Likewise, we define the “new” DNA strands as those detected by label incorporation *after* imposition of fragmenting conditions. We found that in the *seqA recBC* mutant both the old

and the new DNA strands are fragmented to the same extent (10–11%), similar to the overall fragmentation in this experiment (Fig. 6DE), just like in the *rep recBC* mutant (Fig. 6E), where fragmentation is demonstrably consistent with the RF regress-split model (Seigneur *et al.*, 1998), and contrary to the expectations of the RF rear-ending scenario.

To verify that this experiment could in principle detect replication fork rear-ending, we ran the pre- and post-labeling regimen on a strain in which an IPTG-inducible bidirectional replication origin is inserted in the chromosome near *oriC* (the “extra-origin” construct (EOC), to be described elsewhere). Addition of IPTG to a growing culture of this strain causes massive overinitiation of chromosomal replication, increasing ori/ter ratio to 40 within a couple of hours (Fig. 6C) and creating experimental conditions that facilitate replication fork rear-ending into each other. A *recBC* mutant variant of this strain shows massive chromosome fragmentation upon induction of the extra origin (Fig. 6D), while the pre- versus post-labeling protocol reveals that this fragmentation clearly affects the “new” DNA strands more than the “old” ones (Fig. 6E). At the same time, the difference in fragmentation values between the pre-and post-labeling is only 2-fold in the overinitiating strain (Fig. 6E), weakening our conclusion against rear-ending due to the possibility of inadequate sensitivity. We conclude that replication fork disintegration in the *seqA* mutant affects both the old and new strands to the same extent and, therefore, is inconsistent with the replication fork rear-ending scenario, but our experimental system needs to be more sensitive to exclude the rear-ending scenario completely. Overall, we conclude that double-strand DNA breaks in *seqA* mutants are caused by inhibition of replication fork progress as a compensation for overinitiation in the absence of SeqA, but none of the three established models of replication fork disintegration (Fig. 6A) offers a perfect description of the situation, inviting development of a new model.

Discussion

Prompted by our earlier finding that the Δ*seqA* mutants suffer from chromosomal fragmentation, which is lethal in the absence of recombinational repair of double-strand DNA breaks (Kouzminova et al., 2004), in this work we have confirmed this fact genetically, metabolically and physically and then explored the mechanisms behind this fragmentation. We confirmed chromosomal fragmentation genetically by showing dependence of *seqA* mutants on RecBCD and RuvABC, the early and the late functions that help RecA catalyze recombinational repair of double-strand breaks (Kuzminov, 1999, Lukas & Kuzminov, 2006). Other *recA*-colethal mutants are also dependent on RecBC and RuvABC, while being independent of RecF (Bradshaw & Kuzminov, 2003, Kouzminova et al., 2004, Marinus, 2000, Touati *et al.*, 1995). Metabolic confirmation of chromosomal fragmentation in *seqA* mutants was the induction of the SOS response, the regulatory response to reduction of chromosomal replication rates due to high density of DNA lesions (Kuzminov, 1995a, Kuzminov, 1999). We confirmed chromosomal fragmentation physically by quantitative pulsed-field gels electrophoresis and demonstrated by hybridization analysis that it is induced during replication, rather than during the compaction/decompaction or the segregation stages of the chromosomal cycle. The main replication problem of *seqA* mutants turned out to be slow replication forks, especially in the replication origin-centered quarter of the chromosome. Quantitative time course of origin

and terminus increase suggested that inhibition of replication forks is mechanistically linked to the reduction of the replication potential that reigns in overinitiation in the absence of SeqA. Finally, the pre-/post-labeling protocol showed that the old and new DNA strands are fragmented equally in *seqA* mutants, arguing against replication fork rear-ending scenario for chromosomal fragmentation. At the same time, the other two confirmed models of replication fork disintegration, RF collapse and RF regress-split, are also inconsistent with certain aspects of the *seqA* mutant phenotype, inviting a novel mechanism.

Since everything that we have learned so far about replication fork disintegration in *seqA* mutants is generally consistent with the generic “RF breakage” scenario (Fig. 6A, top), the specific defects of the *seqA* mutant cells enable us to formulate a mechanistically explicit version of this idea. The preferential SeqA binding behind replication forks (Waldminghaus et al., 2012) and the accelerated segregation of the newly-replicated loci in *seqA* mutants (Joshi et al., 2013) suggest that SeqA hyperstructure (Norris et al., 2000) organizes and stabilizes the “sister-chromatid-cohesion safety spacer” behind the replication fork (Fig. 1). The sister-chromatid-cohesion phase of the chromosome cycle ensures maturation of nascent DNA via giving enough time for 1) introduction of proper helicity into DNA; 2) joining of Okazaki fragments; 3) repair of DNA lesions, including those requiring pairing with the sister duplex; 4) removal of precatenanes. The sister-chromatid cohesion phase is unusually short in prokaryotes (Nielsen *et al.*, 2006, Nielsen *et al.*, 2007, Vallet-Gely & Boccard, 2013, Viollier *et al.*, 2004), and one of the possible functions of the hypothetical spacer would be to prevent the segregation fork running into the replication fork in case the progress of the latter is inhibited, for example by proteins tightly-bound to template DNA (Fig. 7AB). We propose that in the absence of SeqA the spacer becomes unstable and is reduced by decatenation behind an inhibited replication fork (Fig. 7CD) until the segregation fork runs into the stalled replication fork (Fig. 7E) and breaks it by snapping at a ssDNA region (Fig. 7F). A similar DNA breaking at single-strand regions was proposed to happen during segregation of DNA synthesized in the absence of maturation of Okazaki fragments (Kouzminova & Kuzminov, 2012).

Replication forks in *seqA* mutants are additionally inhibited due to the 2-fold overproduction of the DnaA initiator protein in the absence of SeqA (Camara et al., 2005, von Freiesleben et al., 2000, von Freiesleben et al., 1994), that should cause extra DnaA binding to its multiple sites all over the chromosome. Significant DnaA overproduction or hyperactive DnaA are known to inhibit replication forks even in SeqA+ cells, increasing their ori/ter ratio (Atlung *et al.*, 1987, Løbner-Olesen *et al.*, 1989, Skarstad *et al.*, 1989) and causing chromosome fragmentation (Grigorian et al., 2003, Simmons et al., 2004), while DnaA hypomorph defects are known to accelerate replication forks (Boye et al., 1996). In support of the suspicion that replication forks in *seqA* mutants are generally inhibited by DnaA and nucleoid-binding proteins, we have isolated inactivations of *fis* and *ihfB*, as well as insertions into *dnaA* promoter as suppressors of the *recA*(Cs) *seqA* lethality ((Rotman et al., 2009); ER and AK, to be published elsewhere).

Chromosomal fragmentation is a convenient read-out for lethal DNA damage in bacterial cells, because of the combination of their circular chromosomes and the resolution properties of pulsed-field gel electrophoresis (Khan & Kuzminov, 2013). However, the

uniform appearance of chromosomal fragmentation hides exciting mechanistic complexity. Chromosome fragmentation can happen at all four stages of the chromosome cycle, generating various formats of double-strand ends relative to other ends and to replication forks (Fig. 3A). Fragmentation in non-replicating chromosomes is usually caused by high-energy ionizing radiation (Ward, 1988). It was assumed for some time that the bulk of endogenous fragmentation is caused by replication fork disintegration in damaged DNA (Kuzminov, 1995b, Kuzminov, 1995c, Kuzminov, 1999, Kuzminov, 2011), but recently a clear case of segregation-induced fragmentation in *E. coli* ligase mutants was described (Kouzminova & Kuzminov, 2012), and evidence of condensation-induced chromosome fragmentation in eukaryotes was presented (Pantelias & Terzoudi, 2010). Our line of research facilitates development of tools to induce and characterize chromosomal fragmentation of all kinds, to be able to use this powerful lethal treatment against cancer and pathogenic microbial cells.

Experimental procedures

Growth conditions

Cells were grown in LB (10g Tryptone, 5g Yeast Extract, 5g NaCl, 250 μl 4M NaOH per 1 l) or on LB agar (LB supplemented with 15 g of agar per 1 liter). M9 minimal plates contained 1× M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and were supplemented per 1 liter with 10 mg thiamine (B1), 15 g agar and 2 g galactose or glucose. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml), chloramphenicol (10 μg/ml) and tetracycline (10 μg/ml) were added as needed.

Mutants

E. coli strains used in these experiments were all K-12, and most of them were derivatives of AB1157 (Table S1). Precise deletion-replacement alleles of selected genes were created by the method of Datsenko and Wanner (Datsenko & Wanner, 2000) and confirmed by PCR. Alleles were moved between strains by P1 transduction (Miller, 1972).

SOS induction

To determine the level of SOS induction in the cell, various *seqA* alleles were introduced by P1 transduction into a strain with the MuΔX *cat* construct carrying *lacZ* gene under the *sfiA* promoter (Ossanna & Mount, 1989). When the cells are under SOS-induced stress, either due to mutation or external DNA damage, the promoter is expressed, and the level of β-galactosidase can be quantitatively measured by the modified protocol of Miller (Miller, 1972), using 200 μl of culture (Kouzminova et al., 2004). As a positive control, wild type cells containing the P*sfiA*—>*lacZ* fusion were treated with 100 ng/ml Mitomycin C, a cross-linking agent. At these Mitomycin C concentrations, cells continue slow growth.

Pulsed-field gel electrophoresis

Overnight LB cultures were diluted to OD_{600}=0.02 into 2 ml LB and grown in the presence of 2.5 – 10 μCi of ^{32}P orthophosphoric acid for one hour at 22° and three hours at 37°. All cultures were then brought to OD_{600}=0.35. Cells from 0.5–1 ml aliquots were spun down, washed in 1 ml of TE and resuspended in 60 μl of TE. 2.5 μl of proteinase K (10 mg/ml) and

65 μl of 1.2% agarose in Lysis buffer (see below) was added, and mixed by pipetting. 110 μl of the mixture was then pipetted into the plug mold and allowed to solidify. The plugs were incubated for 1–16 hours at 60°C in lysis buffer (1% sarcosine, 50 mM Tris-HCl, and 25 mM EDTA). Samples were loaded into a 1.0% agarose gel in 0.5× TBE buffer and run at 6.5 V/cm with a pulse time of 90 seconds for 7 hours, 105 seconds for 8 hours, and 125 seconds for 8 hours in a Gene Navigator (Pharmacia) instrument. The gel was vacuum dried onto a piece of chromatography paper (Fisher) for two hours at 80°C and then exposed to a phosphorimager screen until signals from the wells reached between 300,000 and 900,000 counts. If the DNA was unlabeled, the gel was stained for 30 minutes in 0.5 μg/ml ethidium bromide and de-stained for 30 minutes in deionized water before pictures were taken.

Hybridization of the chromosomal smear

Non-radioactive plugs were made and run in duplicate on pulsed field gels as above. After the gel was finished, it was treated for capillary transfer to nylon membrane (Hybond H+). Due to the thickness of the gel, before transfer the plugs were removed and laid on their side. The membrane was divided in two and prehybridized in 5% SDS, 0.5M sodium phosphate pH 7.4 at 65°C for 1 hour. One half of the membrane was probed with ^{32}P-labeled origin-specific probe, while the other half was probed with the terminus-specific probe (Kouzminova & Kuzminov, 2006). Hybridization in the same conditions was overnight; after its completion, the membranes were washed three times in 1/10 strength prehybridization buffer before exposure and quantification by PhosphorImager (FujiFilm FLA-3000).

Analysis of ori/ter ratio

Total DNA was extracted from saturated and exponentially growing cultures by the phenol::chloroform method (Kouzminova & Kuzminov, 2006). 1 μg was denatured in 400 μl 0.1 M NaOH for 15 minutes at 37°C and spotted in duplicate on a positively charged Nylon membrane (Amersham) using a vacuum manifold. After cross-linking, the membrane was divided in two with one half hybridizing to the origin-proximal probe and the other half to the terminus-proximal probe (Kouzminova & Kuzminov, 2006). The spot intensity was measured using the PhosphorImager (FujiFilm FLA-3000), and the ori/ter value was calculated by normalizing to the ori/ter value of a saturated overnight AB1157 culture, which was set to 1.0.

Marker frequency profiling

AB1157 and L-110 strains were grown overnight at 43°C, diluted in the morning to OD 0.1 and continued to be grown at 24°C to OD 0.5. DNA was purified from 2 ml cultures with Wizard Genomic DNA Purification kit (Promega). AB1157 strain grown to OD 0.3, and then treated with 30 μg/ml chloramphenicol for 2 h was used as a reference for aligned chromosome (ori/ter=1). Purified genomic DNA was labeled with BioPrime DNA labelling kit (Invitrogen), as described (Kouzminova & Kuzminov, 2008). The microarray analysis procedure is also described (Kouzminova & Kuzminov, 2008).

Kinetics of origin and terminus replication

L-110 and AB1157 strains were grown overnight in LB at 43°C. Next morning, the cultures were diluted to OD 0.1 and grown at 28°C with shaking. 1 ml aliquots of the growing cultures were taken out at specified ODs to make two agarose plugs. The procedure for making, treating agarose plugs and for agarose plug hybridization was as described (Kouzminova & Kuzminov, 2012). ^{32}P-labelled PCR amplified fragments containing *oriC* and *dif* chromosomal regions were used for hybridization and described (Kouzminova & Kuzminov, 2008). The signals from the plugs at various ODs were measured with the Phosphorimager (FujiFilm FLA-3000) and normalized to the signal of the plug with OD 0.1. The ori/ter ratios in Fig. 5I were derived from the signals used to calculate *ori* and *dif* copy number increase in panels G and H.

Pre-/post-labeling

Strains were grown at 22°C for 20 hours, diluted in fresh LB to yield initial OD of 0.05 and split into three parts for differential labeling. In two parts, ^{32}P orthophosphoric acid was added to a concentration of 0.5–1.0 μCi/ml (for chronic labeling of the culture) or 5–10 μCi/ml (for pre-labeling of the culture). In the third part, destined to be post-labeled culture, no label was added. All cultures were shaken at 22°C until they reach OD of 0.15. At this time, the pre-labeled culture was harvested to remove the label; the pellet was resuspended in the same volume of LB, the cells were pelleted again and suspended again in same volume of LB. After this washing of the pre-labeled culture, the incubation of all cultures was continued at 22°C for another 30 minutes before the label was added to the third part (0.5–1.0 μCi/ml), and all cultures were further split in two sets. For *recBC*(Ts)Δ*rep* strain, one set of the cultures was shaken at 22°C whereas the second set was transferred to 37°C. For *recBC*(Ts)-EOC strain (SRK325), in one set IPTG was added to final concentration of 1 mM, and both sets were transferred to 37°C. The incubation in respective conditions was continued for another 4 hours, after which the cultures were harvested and made into plugs for pulsed-field gel electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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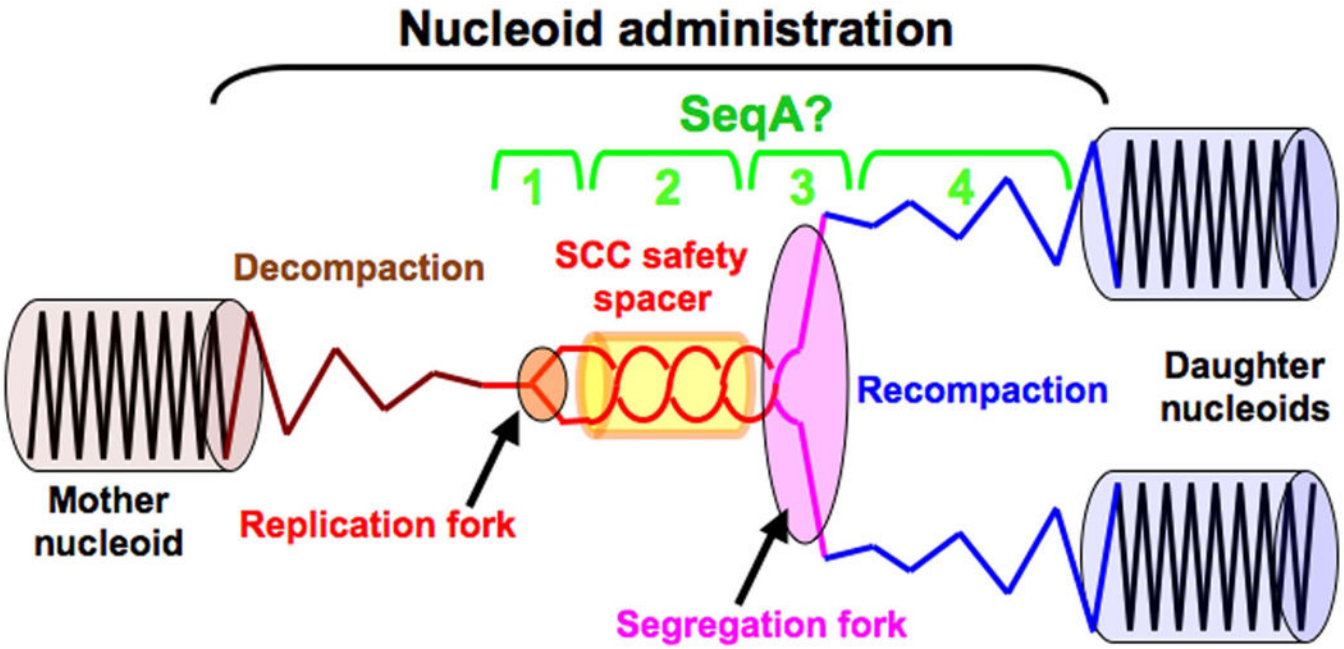

Fig. 1. A scheme of the prokaryotic chromosome cycle

As a result of this set of transactions, duplication of the compacted mother nucleoid (on the left) into a pair of segregated and compacted daughter nucleoids (on the right) is achieved. For simplicity, the chromosome is shown linear, replicated from one end to the other; lines designate DNA duplexes. Distinct stages of the transition, orchestrated by the nucleoid administration, are marked by different colors. SCC, sister-chromatid cohesion. The parts of the chromosome transition at which SeqA may act to avoid chromosome fragmentation are shown by the numbered green half-brackets. For this and subsequent figures, readers should, if necessary, refer to the online (color) version for clarification.

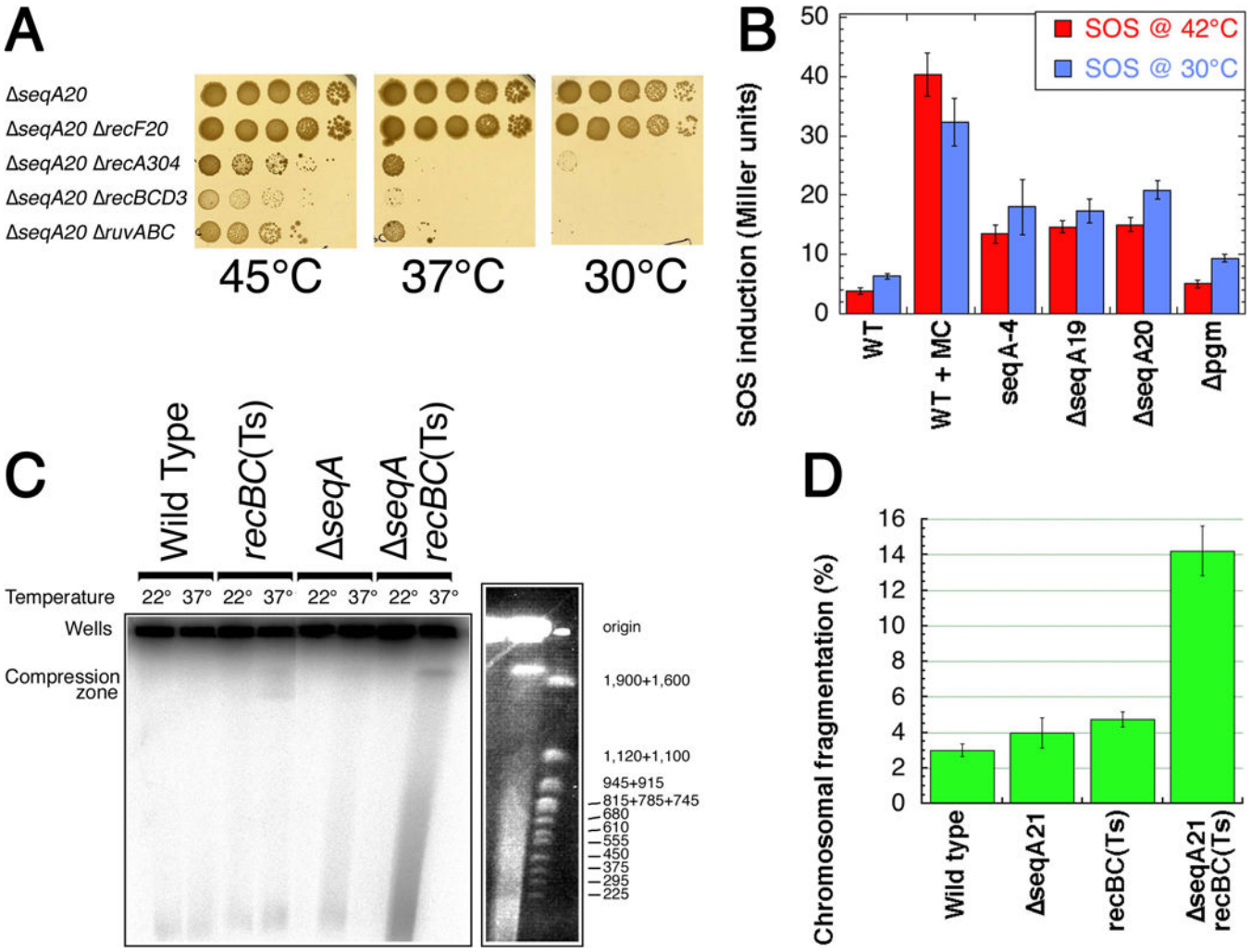

Fig. 2. Rec-dependence, SOS-induction and chromosomal fragmentation in *seqA* mutants

A. The Δ*seqA* mutants depend on RecA, RecBCD and RuvABC, but do not depend on RecF. Cultures, growing at 42°C were serially diluted, spotted by 10 μl on plates, and the plates were incubated at the indicated temperatures for 24 hours. The strains are: Δ*seqA20*, ER15; Δ*seqA20* Δ*recF20*, ER21; Δ*seqA* Δ*recA304*, ER18; Δ*seqA* Δ*recBCD3*, ER19; Δ*seqA* Δ*ruvABC*, ER20. **B.** The level of SOS induction in Δ*seqA* and Δ*pgm* mutant cultures. The corresponding mutants were transduced with a *sfiA::lacZ* fusion construct, and β-galactosidase activity at 30°C versus 42°C was taken as a measure of the SOS induction. As a positive control for SOS induction, wild type cells were treated with sub-inhibitory doses of mitomycin C. The strains are: WT, AK43; WT + MC, AK43 grown in the presence of 100 ng/ml mitomycin C; *seqA-4*, ER70; Δ*seqA19*, ER25; Δ*seqA20*, ER26; Δ*pgm*, ER71. **C.** A representative pulsed-field gel of the ^{32}P-labeled chromosomal DNA isolated in agarose plugs from the indicated strains, grown at the indicated temperatures. The rightmost lane was also stained with ethidium bromide to show its relation to MW markers (yeast chromosomes, size indicated in kbp). Strains are: wild type, AB1157; *recBC*(Ts), SK129; Δ*seqA*, ER15; Δ*seqA recBC*(Ts), ER46. **D.** Quantification of chromosomal fragmentation in the double Δ*seqA recBC*(Ts) mutant. The chromosomal fragmentation was measured as

before, after labeling the chromosomal DNA with ^{32}P-orthophosphate ((Rotman & Kuzminov, 2007) and see “Methods”). The strains are: wild type, AB1157; Δ*seqA21*, ER16; *recBC*(Ts), SK129; Δ*seqA21 recBC*(Ts), ER89. The absolute value of chromosomal fragmentation (%) for any given strain is derived by dividing the signal in the lane (including the compression zone, but excluding the well) by the total signal (lane + well) and multiplying by 100. The level of fragmentation in the double mutant is calculated as follows (Kouzminova & Kuzminov, 2006, Kouzminova & Kuzminov, 2008): (double mutant) — (single mutant #1) — (single mutant #2) + (wild type). For the Δ*seqA21 recBC*(Ts) double mutant, fragmentation thus calculated is 8.5%.

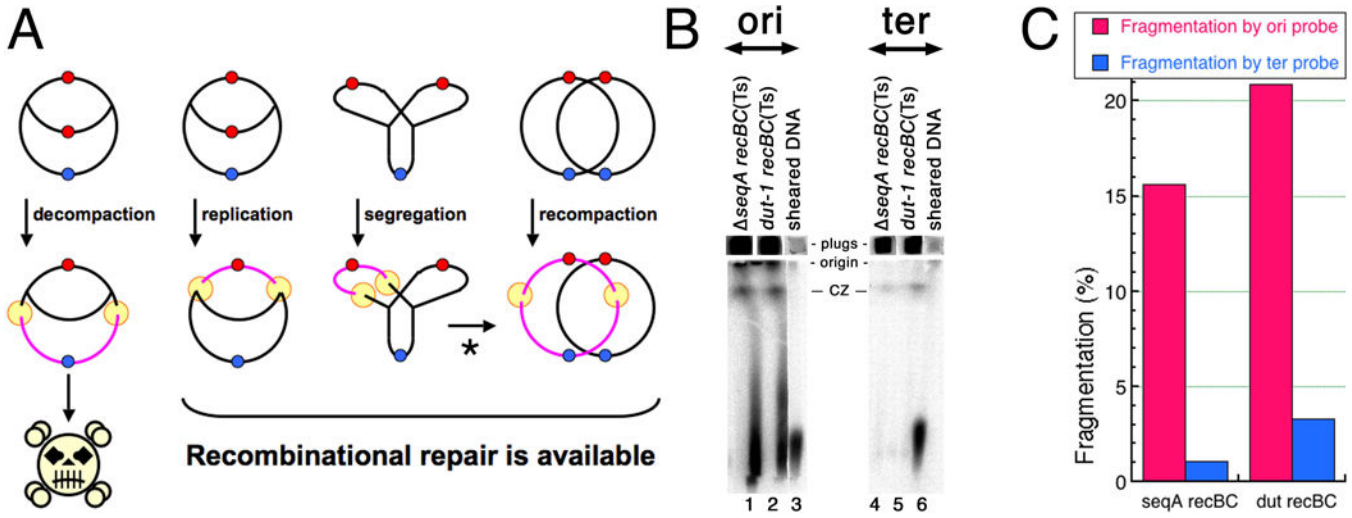

Fig. 3. The nature of chromosomal fragmentation in the Δ*seqA* mutants

A. Double-strand breaks generated during various chromosome cycle transitions: the terminus perspective. Small red circles, replication origins; small blue circles, replication termini, the bigger yellow circles highlight double-strand breaks. Magenta lines indicate linear chromosomal fragments, detectable by PFGE. Replication-induced double-strand breaks are the only one-ended breaks that do not generate chromosomal fragments containing terminus. * Generally, double-strand breaks behind replication forks do not stall the forks in the *recBC* mutants (Kouzminova & Kuzminov, 2006, Kouzminova & Kuzminov, 2008, Kouzminova & Kuzminov, 2012), allowing the broken chromosome to finish replication normally. In contrast to other breaks, decompaction-induced double-strand breaks cannot be repaired (no sister available) and should be lethal. **B.** Pulsed-field gel of fragmenting chromosomes, followed by blot-hybridization with origin-specific or terminus-specific probes. For efficient transfer, the plugs were taken out of the gel after the run and placed flat on the membrane. The *dut recBC*(Ts) strain is shown as a control for the chromosomal fragmentation in which terminus is known to be affected (Kouzminova & Kuzminov, 2006). The sheared DNA (of growing AB1157, aligned with chloramphenicol for 3 hours) serves as a positive control for frank double-strand breaks. The strains are: Δ*seqA21 recBC*(Ts), ER89; *dut-1 recBC*(Ts), AK107. CZ, compression zone. **C.** Quantification of the origin-containing and terminus-containing subchromosomal fragments in the Δ*seqA recBC*(Ts) and *dut-1 recBC*(Ts) double mutants. The values are derived the same way as described in the legend to Fig. 2D. The measured fragmentation values for wild type cells, for two types of single mutants and for the double mutants were averages of 4–8 independent measurements, done on different days, with standard errors comprising from 8 to 33% of the values themselves. We do not have to account for any deviations from the normal ori/ter ratio in *this* procedure, because this is blot-hybridization, and the signal in any given panel is either terminus-specific or origin-specific.

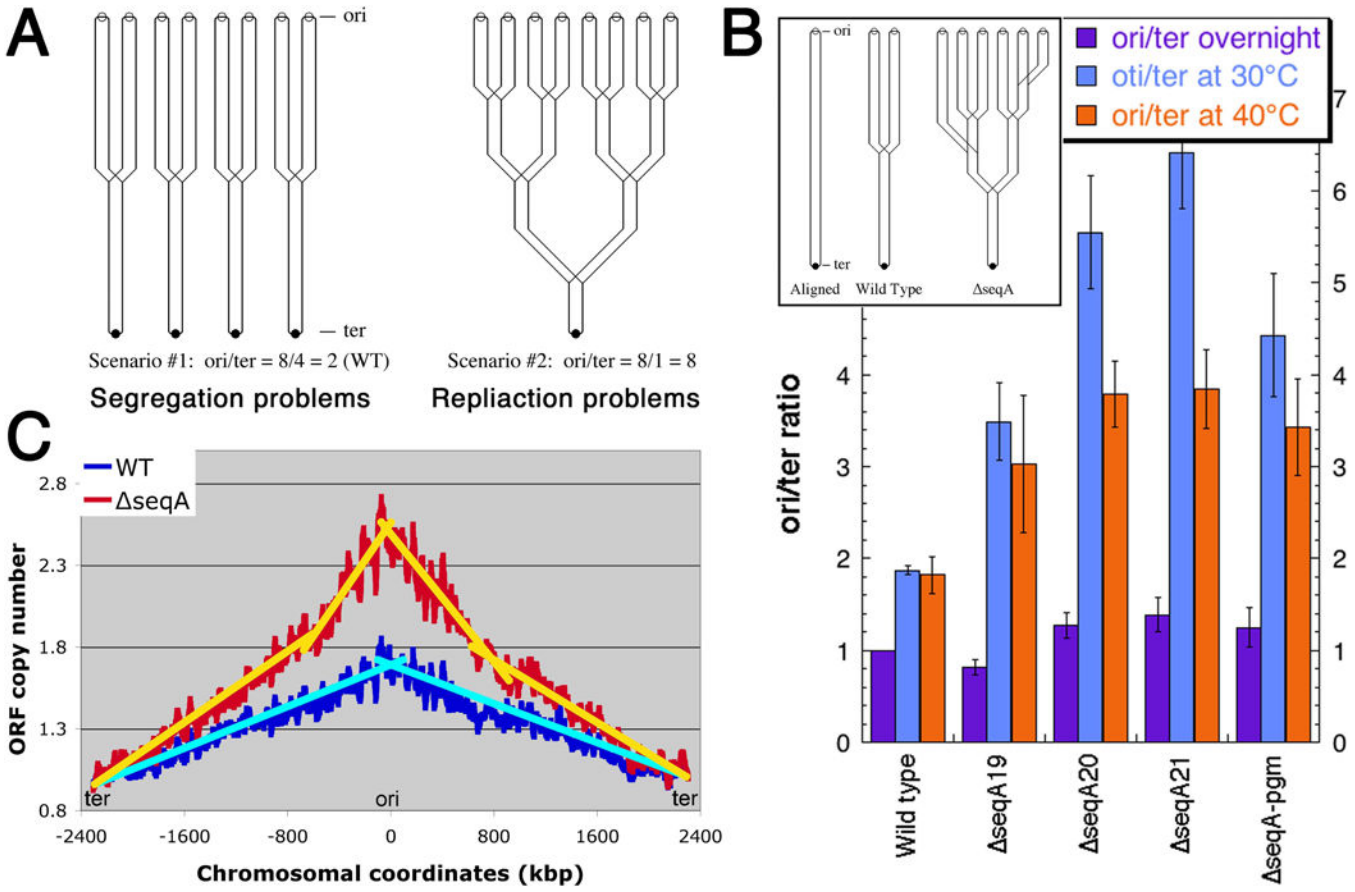

Fig. 4. Replication forks are slow in the *seqA* mutants and accumulate around *oriC*

A. The two possible chromosomal configurations explaining the elevated *oriC* copy number in the *seqA* mutant cells: the partitioning defect (left) versus the replication defect (right), and their numerical expressions in terms of the ori/ter ratios. **B.** The ori/ter ratios in the Δ*seqA* mutants. Total DNA from correspondent strains, either from overnight (saturated) cultures or growing at the indicated temperatures, was deposited on hybridization membrane and hybridized to either origin-specific or terminus-specific probes. The resulting signals were then normalized to the signal from the wild type overnight culture (taken for "1"). The values are averages of five independent determinations, performed on different days, ± standard errors. The strains are: WT, AB1157; Δ*seqA19*, ER17; Δ*seqA20*, ER15; Δ*seqA21*, ER16; Δ*seqA-pgm*, ER47. Inset: the schematic diagram of the chromosome in various cells. From left-to-right: the chromosome, aligned with chloramphenicol (ori/ter = 1); the chromosome in wild type replicating cells (ori/ter = 2); the chromosome in replicating Δ*seqA* mutant cells (ori/ter = 6). **C.** Marker frequency profile of asynchronous cultures of the *seqA* mutant and WT cells. The profiles are normalized to the terminus. Only the trendlines (moving averages of 20 points) are shown. The slopes of trendline segments are highlighted in brighter colors. Strains: *seqA*, L-110; WT, AB1157. We confirm the apparent ori/ter ratio of 1.71 for WT cells and 2.46 for the *seqA* mutant as an artefact of the gene array analysis, since the same DNA samples show ori/ter ratios of 1.73 and 4.85 when hybridized like in "B", still giving the ratio of ori/ter(*seqA*) to ori/ter(WT) ~3.

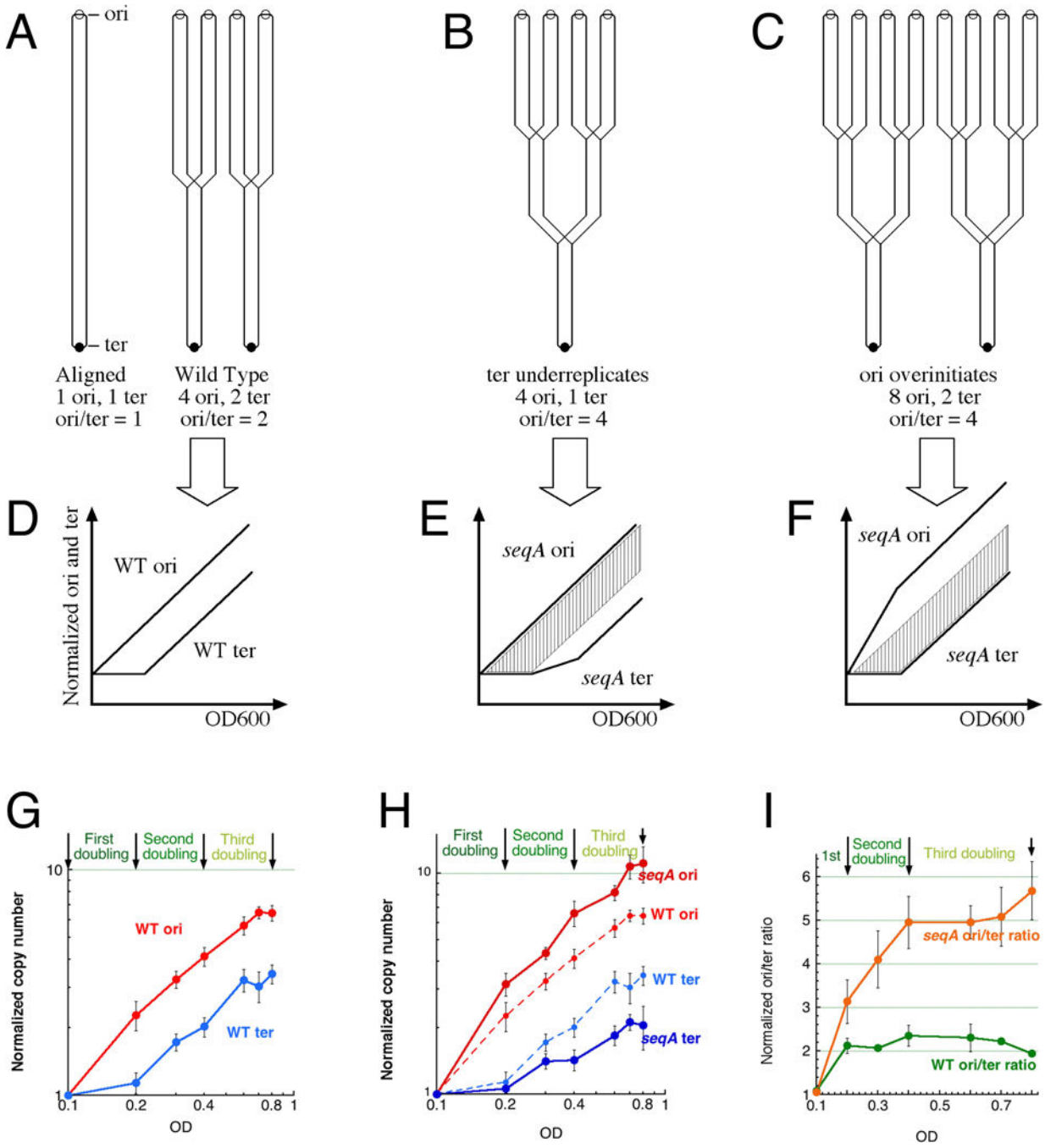

Fig. 5. The *seqA* mutants overinitiate the origin and underreplicate the terminus

A–C. A scheme of normal chromosome replication (A) and two alternative scenarios leading to elevated ori/ter ratios: terminus underreplication (B) versus origin overinitiation (C). **D–F.** The corresponding kinetics of ori and dif increases relative to culture OD the three replication scenarios predict. D, the wild type replication; E, the terminus underreplication scenario; F, the origin overinitiation scenario. In the two theoretical *seqA* mutant graphs, the area delimited by the wild type ori and ter curves is shown stippled. **G.** Origin-DNA and terminus-DNA (*dif*) accumulation from the aligned chromosomes in the wild type cells

(AB1157) as the function of culture density (OD_{600}). Note than both axes are in the log scale. **H.** Origin-DNA and terminus-DNA (*dif*) accumulation from the aligned chromosomes in the *seqA* mutant cells (L-110) as the function of culture density (OD_{600}). Both axes are again in log scale. The WT curves from "G" are shown for comparison. **I.** Ori/ter ratio in the wild type versus *seqA* mutant cells as a function of culture density (OD_{600}). Both axes are in the linear scale in this panel.

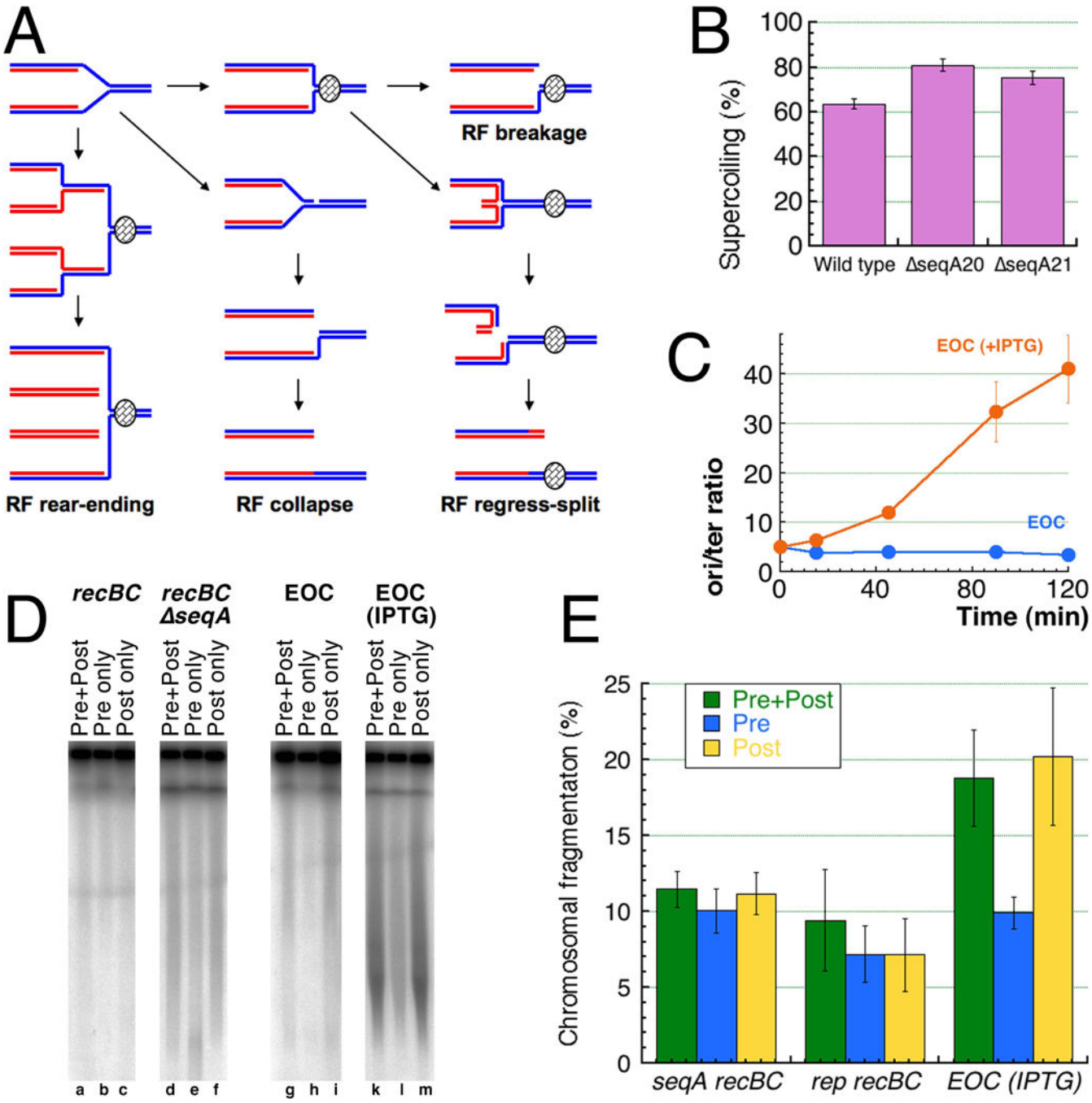

Fig. 6. Testing the models of replication fork disintegration in Δ*seqA* conditions

A. Models of chromosomal fragmentation as a result of replication fork disintegration. The template DNA strands are blue, the newly-synthesized strands are red. The bricked oval denotes a general inhibition of replication fork progress. Note that the rear-ending scenario is the only one that generates fragments made entirely of new DNA strands (no old strand fragmentation). **B.** The fraction of supercoiled plasmid in the total plasmid DNA in the Δ*seqA* mutants is higher than in the wild type cells. The strains are: Wild type, AB1157; ΔseqA20, ER15; ΔseqA21, ER16. The plasmid is pK96. The values are means of seven

independent measurements +/− SEM. **C.** The ori/ter ratio in the extra-origin construct (EOC, IPTG-inducible origin near *oriC*) in the presence (orange line) and absence (blue line) of IPTG. The values are means of three independent measurements +/− SEM (mostly obscured by markers). **D.** A representative pulsed-field gel of the pre-and post-labeling experiment in the *recBC* Δ*seqA* and *recBC* EOC(IPTG) strains. EOC, “extra-origin-construct” strain with IPTG-inducible origin inserted close to *oriC*. The background control strains, correspondingly *recBC* and EOC, are also shown. Strains: *recBC*(Ts), SK129; Δ*seqA21 recBC*(Ts), ER89; *recBC*(Ts) EOC, SRK325. **E.** The level of fragmentation measured with the pre-labeling and post-labeling regiments. “Pre + Post” corresponds to the standard labeling throughout. Strains: *rep recBC*(Ts), AK94; the other strains are like in “C”. The values are means of from three to ten independent measurements +/− SEM.

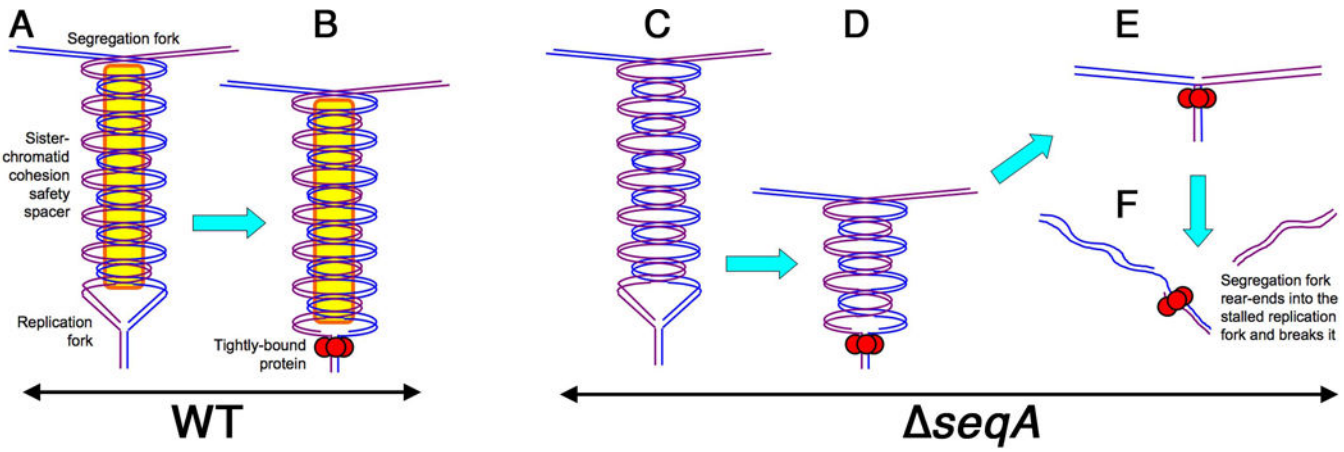

Fig. 7. The model of chromosome fragmentation in *seqA* mutants

For clarity, one of the strands of the original duplex, as well as the corresponding daughter duplex, are shown blue, while the other strand, as well as the corresponding daughter duplex, purple. Yellow rounded rectangle, the SeqA hyperstructure. **A.** Normal replication and segregation in SeqA+ cells. **B.** When replication is inhibited in SeqA+ cells, the SeqA-stabilized SCC safety spacer prevents the segregation fork running into the replication fork. **C.** Normal replication and segregation in *seqA* mutant cells. **D.** When replication is inhibited in *seqA* mutant cells, the now unstable SCC safety spacer is reduced by precatenane removal. **E.** Complete precatenane removal leads to segregation fork slamming into the stalled replication fork. **F.** Replication fork breakage as a result of snapping at a single-strand region due to the pull of the segregation machinery.