Avoiding chromosome pathology when replication forks collide

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Chromosome duplication normally initiates through the assembly of replication fork complexes at defined origins4,5. DNA synthesis by any one fork is thought to cease when it meets another travelling in the opposite direction, at which stage the replication machinery may simply dissociate before the nascent strands are finally ligated. But what actually happens is not clear. Here we present evidence consistent with the idea that every fork collision has the potential to threaten genomic integrity. In *Escherichia coli* this threat is kept at bay by RecG DNA translocase1 and by single-strand DNA exonucleases. Without RecG, replication initiates where forks meet through a replisome assembly mechanism normally associated with fork repair, replication restart and recombination6–8, establishing new forks with the potential to sustain cell growth and division without an active origin. This potential is realized when roadblocks to fork progression are reduced or eliminated. It relies on the chromosome being circular, reinforcing the idea that replication initiation is triggered repeatedly by fork collision. The results reported raise the question of whether replication fork collisions have pathogenic potential for organisms that exploit several origins to replicate each chromosome.

In *E. coli*, the number of head-on fork collisions is kept to a single event by replicating the circular chromosome from a single origin (*oriC*). Chromosome duplication is completed when the two forks established meet in a specialized termination zone (Fig. 1a). This zone is flanked by *ter* sequences bound by Tus protein, forming polar traps that restrict fork movement1. Highly expressed genes are transcribed co-directionally with replication, minimizing the negative impact of conflict between transcription and replication6–8.

Initiation at *oriC* is controlled by DnaA7. However, replication can initiate independently of both DnaA and *oriC*. This stable DNA replication is robust enough to sustain growth in strains lacking RNase HI, especially in minimal media10 (Supplementary Fig. 1). Stable DNA replication is also elevated in strains lacking RecG3, but in this case fork repair, replication restart and recombination6–8, establishing new forks with the potential to sustain cell growth and division without an active origin. This potential is realized when roadblocks to fork progression are reduced or eliminated. It relies on the chromosome being circular, reinforcing the idea that replication initiation is triggered repeatedly by fork collision. The results reported raise the question of whether replication fork collisions have pathogenic potential for organisms that exploit several origins to replicate each chromosome.

![Figure 1](image)

**Figure 1 | PriA triggers DnaA-independent chromosome replication in the absence of RecG.** a, Replichore arrangement of the *E. coli* chromosome. Grey arrows indicate the normal direction of replication and polarity of major transcription. Triangles indicate *ter* sites. b, Genetic analysis of growth without DnaA (*dnaA*46) and in the absence of RecG. The strains were AU1094, AU1091, RCE265, RCE268, AU1066, RCE265, RCE268, N8201, N8205 and N8206. c, Replication profiling of exponential-phase cells. Normalized numbers of reads are plotted against chromosomal coordinate. Sequencing templates for c were from MG1655, N8226, RCE265 and RCE268, all cultured at 42 °C, and for d were from N6576 and JJ1268 cultured at 37 °C.

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wild type. Marker frequency analysis showed an inverted profile for dnaA recG tus rpo* cells at 42 °C, with a peak at the terminus and no evidence of initiation at oriC (Fig. 1c).

Replication initiated in the terminus area in recG cells is abolished by mutations that modulate PriA helicase activity (srgA1, priA300) or which eliminate PriB (Fig. 1d and Supplementary Fig. 4). Both proteins facilitate DnaB loading and replisome assembly at branched DNAs. The same mutations prevent dnaA recG tus rpo* cells from establishing colonies at 42 °C. Eliminating the restart protein PriC does not (Fig. 1b). 5-Bromo-2'-deoxyuridine (BrdU)-labelling confirmed that priA300 reduces stable DNA replication (Supplementary Fig. 4b). Thus the DNA replication initiated at the terminus in cells lacking RecG must be the result of a PriA–PriB-mediated loading of DnaB at a branched DNA structure generated in this region. The ability of srgA1 to prevent initiation (Fig. 1d) indicates that the branched DNA is a 3’ flap. This allele encodes a PriA protein that has specifically lost the ability to unwind such a structure. So 3’ flaps might normally be unwound by RecG and eliminated by single-stranded DNA (ssDNA) exonucleases, reducing the likelihood of PriA targeting the structure and triggering replication.

This fits with the fact that 3’ ssDNA exonuclease activity is needed to keep recG cells alive, and that priA300 overcomes this requirement. We profiled replication in exponential-phase cells lacking ExoI, ExoVII and SbcCD and observed that replication initiates with high frequency in the terminus area despite the presence of RecG (Fig. 2a). This strengthens the notion that replication initiates at 3’ flaps. More importantly, it demonstrates that the re-replication observed without RecG is not a peculiarity of recG cells. Rather, the generation of branched DNA structures with a capacity to trigger replication appears to be a regular feature of the cell cycle triggered each time forks collide to complete replication.

We investigated what happens when forks are prevented from colliding by using strains in which the chromosome is linearized by phage N15 telomerase at a tos site inserted near difC (Supplementary Fig. 5). Linearization has no effect on the growth of wild-type cells (Supplementary Fig. 5b). However, it abolishes the ability of dnaA recG tus rpo* cells to establish colonies at 42 °C and reduces amplification of the terminus area (Fig. 2b–d). The residual amplification observed depends on forks established at oriC (Fig. 2c and Supplementary Fig. 6), indicating that it is most probably the result of some replication through the hairpin (Supplementary Fig. 5a and Supplementary Discussion). Amplification is evident on both sides of the tos site (Fig. 2d), making it unlikely that it is due to activation of a dormant origin or of a hotspot for initiation at R-loops.

But why should fork collisions trigger initiation? We have proposed that when replisome complexes meet, the DnaB helicase of one fork often displaces the nascent leading strand of the opposing fork, generating a 3’ flap. DnaB would most probably collide with and dislodge the leading strand polymerase of the opposing fork to which the 3’ end of the nascent leading strand is engaged (Fig. 3a and Supplementary Discussion). This flap would be degraded by 3’ ssDNA exonuclease or converted by RecG to a 5’ flap and subsequently

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**Figure 2 |** Replication fork collisions trigger DnaA-independent replication.

- **a.** Replication profile of exonuclease-depleted cells. Sequencing templates were from exponential-phase N6953 cultured at 37 °C. **b.** DnaA-independent growth. The strains analysed were RCe268, RCe384, RCe385 and RCe387.
- **c.** BrdU labelling of a fragment of NotI-digested DNA (b) located near the replication terminus relative to a distant reference fragment (4a). The strains were RCe405, RCe409 and RCe418. Cultures of dnaA46 strains of the genotypes indicated were pulse labelled with BrdU at the indicated times after the shift to 42 °C.
- **d.** Replisome profiling showing effect of chromosome linearization on replication. Sequencing templates were from N8226, RCe391 and RCe399 cultured at 37 °C.

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Eliminating just the ExoV activity of RecBCD has no such effect is prevented by inactivating RecA, the RuvABC Holliday junction resolvase or the RecBCD recombinase (Fig. 3b and data not shown). Eliminating just the ExoV activity of RecBCD has no such effect (Fig. 3b, recD derivative). Marker frequency analysis confirmed that inactivating RecB prevents over-replication of the terminus. Indeed, marker frequency is reduced in this region (Fig. 3c). This is a feature of cells lacking RecBCD as it is also seen in a recB single mutant (Supplementary Fig. 7a). BrdU incorporation confirmed that inactivating RecB reduces stable DNA replication in recG cells (Supplementary Fig. 7b). Taken together, these data indicate that without RecG, fork collisions often lead to the generation of dsDNA ends that are then targeted by RecBCD. The viability of recG recB double mutant cells5 excludes the possibility that these ends are the result of fork or chromosome breakage.

Profiling cells carrying two copies of oriC (Fig. 3d) showed that fork collisions trigger initiation of replication wherever forks meet. As expected17, both origins fire with equal efficiency on a population basis (Fig. 3e), leading to the termination of replication in two distinct zones, one in the area flanked by ter sites and a second in the shorter of the two intervals between the origins. The latter is shallower and less focused, which is to be expected given the absence of Tus/ter traps, whereas the former is deeper and more sharply defined. There is also a clear step between terA and terB, consistent with the clockwise fork from oriZ being blocked by Tus bound at terB, having reached this point before meeting the anticlockwise fork from oriC.

In the recG derivative, we observed a strong increase in amplification of the terA–terB interval (Fig. 3e and Supplementary Discussion). More notably, the termination region in the shorter interval is also noticeably shallower. This is precisely what one might expect if fork collisions were prone to trigger initiation. Without Tus/ter complexes to focus events, such initiation would lead to a wide region of amplification rather than to a distinct peak, as is seen when Tus is eliminated from a recG strain (cf. Figs 3c and 4a). Incomplete synchronization of origin firing17 would also reduce collisions in this region. These data support the notion that replication fork collisions have the potential to trigger new replication wherever forks meet.

But how is replication maintained in dnaA recG tus rpo* cells without oriC firing? Shifting the cells from 30 °C to 42 °C would not affect existing forks. When these collide at the terminus and trigger initiation, the new forks would proceed towards oriC. Any subsequent fork collisions would create an opportunity to repeat the cycle, potentially ad infinitum. With no Tus/ter complexes, collisions might become increasingly random. However, a broad peak of increased terminal markers is evident (Fig. 1c), suggesting perhaps that initiation is not random. Therefore, it is notable that the replication profile of tus cells in exponential phase is identical to wild type (Fig. 4a, b). Even in recG tus cells the termination area is still evident, although more broadly delineated (Fig. 4a). Thus, the sharp focusing of termination in wild-type cells is probably due to some factor in addition to Tus/ter. We suggest that the polarity of transcription impedes forks moving beyond the terminus towards oriC. Broadening of the terminus area in tus rpo* cells supports this idea (Fig. 4b). However, rpo* is unlikely to eliminate this problem entirely. Therefore, dnaA recG tus rpo* cells with forks moving towards oriC, having duplicated the terminus, might be overrepresented, giving the marker frequency observed (Fig. 1c). Also, if forks moving towards oriC failed to complete duplication of the chromosome because of the conflicts with transcription, DNA from the affected (dead) cells would further bias the profile in favour of terminal markers.

Without means to control initiation, these cells lack the ability to coordinate chromosome replication with cell growth. New rounds of replication initiate only after replication fork collision, preventing the increased rate of growth and division made possible in wild-type cells by firing oriC before the previous round of replication has been completed4. So, what happens when growth ceases? Marker frequency analysis of recG cells grown to saturation showed an overrepresentation of the terminus (Fig. 4c), as if they had ceased growing with forks stalled at Tus/ter. An even broader elevation of terminal markers is observed in saturated recG tus cells. Coupled with its absence in tus cells (Supplementary Fig. 8), this persistence of forks suggests that once stationary-phase dnaA recG tus rpo* cells are diluted in fresh growth medium, replication resumes at the stalled forks.

Taken together, the studies reported here demonstrate that RecG and 3′ ssDNA exonucleases play critical roles in limiting re-replication of the already replicated DNA. It is notable that re-replication is blocked by mutations (priA300, srqA1 and AprIB) that suppress the recG mutant phenotype16. This suggests that such replication has pathological consequences that destabilize the genome.

The single fork collision typical of the average E. coli cell cycle is a stark contrast to the multiple events occurring in eukaryotes. Recent studies have shown that the final stages of replication in eukaryotic cells have pathogenic potential19,20. RecG is absent, but several studies have reflected on the ability of human and yeast helicases to remodel branched DNA structures in a manner reminiscent of RecG21–24. Several ssDNA exonucleases have also been linked with replication, both nuclear and mitochondrial25. The human mitochondrial nuclease MGM-E1, loss of which is associated with multi-systemic mitochondrial disease, has been shown to process displaced ssDNA flaps26. So, as with E. coli, eukaryotic helicases and ssDNA exonucleases may well turn out to have important roles in making sure that replication is completed without triggering pathological events that destabilize the genome. Indeed, limiting the incidence of pathological events at termination may prove to be as crucial to genome stability as the prevention of re-initiation at replication origins2.

**METHODS SUMMARY**

_E. coli_ strains are described in Supplementary Table 1. The dnaA46 allele encodes a thermosensitive DnaA protein that is inactive at 42 °C. Genetic manipulations, molecular procedures and methods for assessing growth and viability followed established protocols (see Supplementary Material). Replication profiling by
marker frequency analysis used AB SOLID sequencing to measure sequence copy number. Enrichment of uniquely mapping sequence tags, in 1 kb windows, was calculated for an exponentially growing (replicating) sample relative to a non-replicating stationary-phase wild-type (MG1655) sample to correct for differences in read depth and to allow presentation of the data as a normalized marker frequency\(^*\).

**Full Methods** and any associated references are available in the online version of the paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Information** Deep sequencing data have been deposited with NCBI Gene Expression Omnibus under accession number GSE41975. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.J.R. (christian.rudolph@brunel.ac.uk).
METHODS

Bacterial strains and general methods. The E. coli strains used in this study are derivatives of E. coli K-12 MG1655 unless stated otherwise (Supplementary Table 1). The dnaA46 allele encodes a thermosensitive DnaA protein that is inactive at 42 °C. The oriC deletion allele tagged with a sequence encoding resistance to kanamycin (oriC::kan) was generated by the single-step gene replacement method. Luria and Burrous broth and 56/2 salts media have been cited elsewhere. Strain constructions, genetic manipulations and assays for bacterial and phage growth used standard microbiological materials and protocols, as described or cited. For assessing growth without DnaA, cultures of E. coli K-12 MG1655 were diluted 400-fold in fresh broth and incubated with vigorous aeration until A650 reached 0.4 at the temperature indicated. Samples from these exponential-phase cultures were frozen in liquid nitrogen at this point for subsequent DNA extraction. Incubation of the remaining cultures shifted to 42 °C. The A650 reached 0.2 before spotting 10 μl samples of each dilution on LB agar. Duplicate plates were incubated at 30 °C and 42 °C. Pictures were taken after 24 h unless stated otherwise.

Replication profiling by marker frequency analysis. Samples from cultures of a strain grown to A650 ~ 1.2 in LB broth were diluted 400-fold in fresh broth and incubated with vigorous aeration until A650 reached 0.4 at the temperature indicated. Samples from these exponential-phase cultures were frozen in liquid nitrogen at this point for subsequent DNA extraction. Incubation of the remaining culture was continued until several hours after the culture had saturated and showed no further increase in A650. A further sample (stationary phase) was frozen at this point. DNA was then extracted using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Marker frequency analysis used AB SOLiD sequencing for differences in read depth across the genome and to allow presentation of the data as a marker frequency, as described previously.

BrdU labelling. BrdU labelling and detection by immunostaining was essentially as described. Cells were grown in 56/2 salts supplemented with 0.2% casamino acids and 0.32% glucose to A660 nm = 0.2. The culture was split into various 2 ml aliquots, BrdU (Sigma) was added to the first aliquot to 20 μg ml⁻¹ and the cultures shifted to 42 °C. At the times indicated, BrdU was added to one of the remaining aliquots. The aliquots were labelled with BrdU for 8 min, pelleted and re-suspended in 85 μl TEE buffer (10 mM Tris-HCl, 10 mM EGTA, 100 mM EDTA, pH 8.0) containing 0.05% lauroylsarcosine and 0.5% SDS. Eighty-five microlitres of liquid 1.4% low melting point agarose was added and the mixture solidified in a disposable plug former (Bio-Rad) at 4 °C. Plugs were treated with 10 mg ml⁻¹ lysozyme in 3 ml TEE buffer containing 0.05% lauroylsarcosine and 0.5% SDS for 2 h at 37 °C and then at 52 °C overnight with 5 mg ml⁻¹ proteinase K in 3 ml TEE containing 1% SDS. Plugs were washed in TEE for 30 min at 37 °C, treated with 1 mM phenylmethane sulphonyl fluoride (freshly prepared as 100 mM stock solution in methanol) in fresh TEE for 1 h at 37 °C, washed in fresh TEE for 30 min at 37 °C and finally in 0.1 × TEE for 30 min at 37 °C. The plugs were subsequently transferred into 300 μl restriction enzyme buffer and incubated for 30 min at room temperature, the buffer changed and 25 units of NotI (NEB) added. Chromosomal DNA was digested overnight and the fragments separated on a 0.8% agarose gel (Bio-Rad pulse field certified agarose) in 0.5 × TBE using a CHEF Mapper PFGE system (Bio-Rad), running with a gradient voltage of 6 V cm⁻¹, an included angle of 120 °, and initial and final switch times of 1.65 and 32.45 s, respectively, with a run time of 20 h at 14 °C. DNA was transferred to a Hybon-N+ Membrane (GE Healthcare) by alkaline vacuum transfer and ultraviolet crosslinked (120 mJ cm⁻²). Blocking was achieved with TBS Tween (50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.5% Tween 20) containing milk powder (2%). After blocking the membrane was incubated for 2 h in the presence of mouse anti-BrdU antibody (Santa Cruz) and diluted 1:5,000 in TBS Tween. Horseradish peroxidase-conjugated secondary antibody (goat anti-mouse, Bio-Rad) was used at a dilution in TBS Tween of 1:10,000 for 1.5 h. The membrane was incubated with ECL Plus Western Blotting Detection Reagents (GE Healthcare) and the signal visualized either by exposure to X-Omat UV Plus film (Kodak) or by the ChemiDoc chemiluminescence detection system (Bio-Rad).

Chromosome linearization. Linearization of the E. coli chromosome was achieved using the bacteriophage N15 telomere generating system (Supplementary Fig. 5). N15 is a temperate E. coli phage, which does not integrate into the chromosome during lysogenization. Instead, the N15 telomerase, TeN, cleaves and processes a specific phage DNA sequence called tos, generating a linear chromosome with hairpin ends (Supplementary Fig. 5). When replication forks reach these structures, the nascent leading and lagging strands are sealed, forming a chromosomal dimer. This restores two tos sites, which are immediately re-processed by TeN, thus allowing segregation of two linear chromosomes. For our studies, we exploited E. coli derivatives that carried a tos site inserted near the dif locus, close to terC. These were lysogenized with phage N15 to linearize the chromosome (Supplementary Fig. 5). Preparation of phage N15 stocks and E. coli lysogens followed methods and protocols described for phage λ.