Title

The bacterial MRE11-RAD50 and DNA2-WRN homologs process replication forks at distinct and separate loci on the chromosome

Raymond L. Spolek, Paden Y.H. Christian, Charmain T. Courcelle & Justin Courcelle*
Dept. of Biology, Portland State University, Portland Oregon

*Correspondence: justc@pdx.edu

Key words: MRE11-RAD50, SbcC-SbcD DNA2-WRN, RecJ-RecQ, DNA replication

Running title: SbcC-SbcD RecJ-RecQ process forks at distinct loci

Abbreviations: 2-D, Two-dimensional; IdU, 5-iodo-2'-deoxyuridine; CldU, 5-chloro-2'-deoxyuridine; DGCthy, Davis media supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine; LBthy, Luria-Bertani media supplemented with 10 μg/ml thymine; OD, optical density; EDTA, ethylenediaminetetraacetic acid; NaCl, sodium chloride; Tris, 2-Amino-2-(hydroxymethyl)propane-1,3-diol; UV, ultraviolet; dCTP, deoxycytidine triphosphate.

Abstract

Human BRCA2 protects the DNA when replication forks stall, whereas MRE11-RAD50 and WRN-DNA2 process or partially degrade these substrates. When mutated, these genes result in distinct genetic instabilities and cancers, arguing they have unique, not redundant, functions.

Escherichia coli encodes functional homologs of MRE11-RAD50 (SbcC-SbcD), WRN-DNA2 (RecQ-RecJ), and BRCA2 (RecF). Here, we use 2-dimensional gels, pulse-labelling, and replication-profiling analysis to show the bacterial homologs act at distinct substrates and loci on the chromosome. Whereas RecF and RecJ-RecQ protect and process DNA at arrested replication forks to facilitate repair, RecBCD and SbcC-SbcD protect and process DNA at sites where forks converge. Comparing the assays used in *E. coli* to human cells, we consider whether these cellular roles may be functionally conserved.

Introduction

Accurate duplication of the genome requires that cells initiate, elongate, and then complete replication to ensure that each daughter cell inherits an identical copy of the genetic information. During this process, replication forks encounter a variety of hurdles that hinder their progression through the genome. These include lesions such as abasic sites; small base modifications due to depurination or oxidation like uracils, thymine glycols, or 8-oxo-guanines; bulky DNA adducts due to UV or chemical exposure; interstrand DNA crosslinks; single- and double-strand DNA breaks resulting from ionizing radiation; and physical barriers such as DNA secondary structures or DNA-bound proteins or RNA (reviewed in [1]). These common biological events impose structurally diverse challenges that can prevent the translocation of replisomes through DNA in different ways. Restoring replication upon encountering these

qualitatively distinct impediments is likely to require different subsets of repair enzymes and utilize distinct pathways.

A central player in human cells for maintaining arrested replication forks is BRCA2, a protein frequently mutated in hereditary breast and ovarian cancers, but also associated with pancreatic, brain and other cancers [2–4]. Following replication disruption, the protein mediates loading of a RAD51 nucleoprotein filament on the single-stranded regions at the fork [3, 5]. RAD51 acts by pairing single-strand DNA with homologous duplex DNA [6], an activity required during meiosis for bringing together corresponding maternally and paternally inherited chromosomes, but also during replication of mitotic cells for pairing sister chromatids at replication forks when progression is impeded. At arrested replication forks, the pairing serves to protect the nascent DNA from extensive exonucleolytic degradation, and is required for replication to resume normally [3, 5, 7, 8].

Several additional enzymes have been identified that are present or recruited to stalled or disrupted replication forks, contributing to the protection [9–12]. Among this class are some that partially degrade or process the nascent DNA at stalled replication forks, and include the WRN-DNA2 helicase-nuclease, and the MRE11-RAD50 structure-specific nuclease [3, 13, 14].

WRN and DNA2 encode a RECQ-family helicase and a 5'-exonuclease, respectively, although both proteins individually possess cryptic helicase and nuclease activities [15–17]. WRN functions in concert with DNA2 to partially degrade the DNA at forks stalled or damaged by hydroxyurea treatment through a mechanism that is stimulated by interactions with single-strand binding protein, RPA [13, 16, 18]. The processing is required for the timely resumption of DNA synthesis, making it likely the degradation represents a normal part of the recovery process [13, 19, 20]. Mutations in WRN result in Werner's syndrome, a rare genetic disorder manifesting as premature aging or progeria [21]. Patients are predisposed to thyroid cancers, melanoma, meningioma, soft tissue sarcomas, osteosarcomas, and colorectal cancer (reviewed in [22]). DNA2 is essential for viability and is upregulated in a wide range of cancers containing TP53 mutations which is associated with poorer outcomes [15, 23].

MRE11-RAD50 is a heterotetrameric complex with a cohesin-like architecture that is also capable of processing and partially degrading DNA at hydroxyurea-stalled replication forks [3, 13, 24]. The complex contains a prominent endonuclease activity that incises DNA hairpin-like structures, as well as double-stranded 3'-5' exonuclease activity [17, 25–28]. MRE11-RAD50-mediated degradation is predominantly detected in the absence of BRCA2, making it unclear if the processing is associated with the normal recovery process or represents an aberrant condition [3, 29–31]. Irrespective, the protein complex has a critical cellular role. Inactivation of this enzyme complex renders cells hypersensitive to double-strand breaks, impairs cell cycle progression, and leads to large palindromic amplifications and widespread genetic instabilities [32–37]. In humans, null mutants in MRE11 or RAD50 are embryonic lethal, and hypomorphs are associated with the hereditary genetic disorders ataxia telangiectasia-like disorder (ATLD), and Nijmegen breakage syndrome-like disorder (NBSLD) [33, 38–40]. Somatic mutations can be associated with ovarian, breast, and glioma cancers, and affect prognosis and survival rates (reviewed in [41]).

The mechanism by which replication is restored has also come into focus as a promising target for cancer therapies, with synthetic lethal approaches clinically validated by the efficacy of PARP inhibitors in treating BRCA2 cancers and WRN inhibitors in treating cancers with

microsatellite instabilities (reviewed in [42, 43]). Both replication and repair are highly conserved across evolutionarily divergent organisms. Our mechanistic understanding of BRCA2 and WRN began in *Escherichia coli*, where RecF and RecQ are considered functional homologs. In the case where replication is disrupted by UV-induced damage, RecF, together with RecO and -R, facilitate loading of a RecA filament at the replication fork, similar to BRCA2 loading RAD51 [44–46]. This loading is required to protect and maintain the structural integrity of the arrested fork DNA [47–50]. Additionally, RecJ together with RecQ, a 5'-3' single-stranded exonuclease and 3'-5' helicase, partially degrade the nascent DNA at times before DNA synthesis resumes through a mechanism stimulated by interactions with single-strand binding protein, similar to WRN-DNA2 [49, 51–55]. In *E. coli*, the nascent degradation has been shown to preferentially occur on the nascent lagging strand of the fork, which is thought to create a substrate for RecF, -O, -R, and RecA to bind and stabilize, as well as restore the lesion-containing region to a double-stranded form, which is essential for nucleotide excision repair to bind the lesion and effect repair [48, 49, 51, 56]. Similar to WRN-DNA2, when either RecJ-RecQ processing or excision repair cannot occur, DNA synthesis fails to recover normally [52]. Under these conditions in *E. coli*, the recovery is delayed, and survival becomes dependent on translesion synthesis by polymerase V [52].

E. coli also has clear structural and functional homologs of MRE11-RAD50, encoded by *sbcC* and *sbcD*. The genes were originally identified as mutations which suppressed the poor growth and DNA damage sensitivity of strains lacking both RecBCD and Exonuclease I [57, 58]. Subsequent characterizations demonstrated that the genes prevented large palindromes from persisting in the bacterial DNA, an observation that mimics the genetic instabilities arising in the absence of the eukaryotic homologs [35, 59]. Recent work in *E. coli* has shown that both SbcC-SbcD and Exo I process DNA at loci where two replisomes converge [60–62]. The processing is required for chromosome replication to complete normally, and mutants lacking these genes depend on an aberrant form of recombination to maintain viability that leads to genetic instabilities at these loci [61].

However, whether the bacterial SbcC-SbcD functions at replication forks disrupted by DNA damage has not been examined. Considering the intensive focus on replication processing of DNA damage and Mre11-Rad50 as a potential therapeutic target, this represents an important gap in our state of knowledge to address. While both WRN-DNA2 and Mre11-Rad50 are thought to process stalled replication forks, mutations in these genes lead to distinct phenotypes with respect to the genetic instabilities, cancers, and genetic disorders that arise. These observations would argue that their functions are likely to be distinct, rather than redundant with respect to replication fork processing. Here, we show that the bacterial homologs of these eukaryotic enzymes function at distinct and separate events on the *E. coli* chromosome.

Materials and Methods

Strains and plasmids

All strains used in this work are derived from SR108, a *thyA deoC* derivative of W3110 [63]. HL944 (SR108 *recQ1803*::Tn*3*), HL924 (SR108 *recJ284*::Tn*10*), CL579 (SR108 *recF6206*::*tet*), CL1056 (SR108 *recBCD*::*cat*), and CL628 (SR108 *recF332*::Tn*3 recQ6215*::*cat*) have been described previously[49–51, 61].CL5475 (SR108 *recJ*::FRT-kan-FRT) and CL5478 (SR108

recF6206::*tet recJ*::FRT-kan-FRT) were constructed by P1 transduction of the *recJ*::*kan* allele from JW2860 [64] into SR108 and CL579, respectively, and selecting for kanamycin resistance. CL5473 (SR108 *sbcC*::FRT-kan-FRT) and CL5476 (SR108 *recF6206*::*tet sbcC*::FRT-kan-FRT) were made by P1 transduction of the *sbcC*::*kan* allele from JW0387 [64] into SR108 and CL579, respectively, and selecting for kanamycin resistance. CL4276 (SR108 *recBCD*::*cat sbcCD*::*gent*) was made by P1 transduction of *recBCD*::*cat* allele from CL1056 into CL2344 [61], selecting for chloramphenicol resistance. CL5541 (SR108 *recJ284*::Tn*10 recBCD*::*kan*) was constructed by P1 transduction of *recBCD*::*kan* allele from KM21 [65] into HL924, selecting for kanamycin resistance. pBR322 contains a ColE1 origin of replication [66].

UV survival

Fresh overnight cultures were diluted 1:100 in Davis media supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thymine (DGCthy) and grown at 37°C to an optical density of 0.4 at 600 nm (OD_{600}). At this time, 100-μl aliquots were removed from each culture and serially diluted in 10-fold increments. Triplicate 10-μl aliquots of each dilution were spotted onto Luria-Bertani agar plates supplemented with 10 μg/ml thymine (LBthy) before the plates were UV irradiated at the indicated dose using a 15-watt germicidal lamp (254 nm, 1 J/m^2 per sec at the sample position). Plates were incubated at 37°C overnight and viable colonies were counted the next day to determine the surviving fraction.

Nascent DNA Degradation

Overnight cultures were diluted 1:100 and grown in DGCthy to an OD600 of 0.4 in a 37°C shaking water bath. Cultures were then pulse labeled with [^{3}H]thymidine (1 μCi/10 μg/mL) for 10 s, filtered on Whatman 0.4-μm membrane filters, and washed twice with 3 mL of cold NET buffer (100 mM NaCl/ 10 mM EDTA, pH 8.0/10 mM Tris, pH 8.0). The filter was then resuspended in prewarmed nonradioactive DGCthy media, UV irradiated with 40 J/m^2, and incubated at 37°C. At the times indicated, duplicate 200-μL aliquots of cells in culture were lysed, the DNA was precipitated by the addition of 5ml cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amount of ^{3}H on each filter was determined by scintillation counting. Duplicate samples were averaged for each experiment and the fraction of radioactivity remaining was then determined by dividing the average of the time point by the amount observed at time zero. Plots represent the average of at least three independent experiments and error bars were plotted by determining 1 standard error.

2-D agarose gel analysis of plasmid replication intermediates

Overnight cultures of cells containing plasmid pBR322 were diluted 1:100 in DGCthy medium containing 100 μg/ml ampicillin and grown in a 37°C shaking water bath to an OD600 of 0.5. At this time, 0.75-ml of cultures were mixed with equal volumes of ice-cold 2× NET (100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA). The remaining cultures were UV irradiated at 50 J/m^2, then returned to 37°C and at the indicated times post-UV irradiation, 0.75-ml aliquots were mixed with equal volumes of ice-cold 2× NET. Cells were pelleted and frozen at −80°C. Samples were resuspended in 140 μl of lysozyme (1 mg/ml) and RNaseA (0.2 mg/ml) in TE (10 mM Tris, pH 8.0, 1 mM EDTA) and lysed for 30 min at 37°C. Then Sarkosyl (10 μl of 20% [wt/wt]) and Proteinase K (10 μl of 10 mg/ml) was added and incubation continued for 60 min. Samples

were then serially extracted with two volumes phenol-chloroform (1/1) and then again with two volumes chloroform followed by dialysis for 1 h on 47 mm MF-Millipore 0.05-µm pore disks (Merck Millipore #VMWP04700) which were floated on a 250-mL beaker of TE (1 mM Tris, pH 8.0, 1 mM EDTA). The total genomic DNA was digested with PvuII (New England BioLabs) which restricts the plasmid near its origin of replication. For the first dimension, samples were extracted with one volume of chloroform before equal cell equivalents were electrophoresed in a 0.4% agarose gel in 1× TBE at 1 V/ cm for 15 h. For the second dimension, the lanes were excised, rotated 90°, and recast in a 1% agarose gel in 1× TBE and electrophoresed at 6.5 V/cm for 6.5 h. For the Southern analysis, DNA in the gels was transferred to a Hybond N+ nylon membrane, and the plasmid DNA was detected by probing with either ^{32}P-labeled pBR322 or pCL01 plasmid DNA prepared by random-primer labeling (Agilent Technologies) using α^{32}P-labeled-dCTP (3000 Ci/mmol; PerkinElmer) and visualized using a STORM PhosphorImager with its associated ImageQuant analysis software (Amersham Biosciences).

Replication profiles

Cultures grown overnight were diluted 1:250 in fresh LBthy media. All cultures were grown at 37°C with aeration. To normalize profiles, stationary-phase cultures were grown for 36 h before harvesting. When cultures reached an OD600 of 0.4, genomic DNA was purified by placing 0.75-mL of culture into 0.75-mL ice-cold 2X NET buffer (100 mM NaCl, 10 mM Tris at pH 8.0, 10 mM EDTA). All samples were then pelleted by centrifugation, resuspended in a solution containing 140 µl of 1 mg/ml lysozyme and 0.2 mg/ml RNaseA in TE (10 mM Tris at pH 8.0, 1 mM EDTA), and incubated at 37°C for 30 min to lyse cells. Subsequently, Sarkosyl [10 µl, 20% (wt/wt)] and Proteinase K (10 µl, 10 mg/ml) were added, and the incubation was continued at 37°C for an additional 30 min. The samples were then further purified by extracting the DNA with 4 vol phenol/chloroform (1/1) followed by dialysis for 1 h using 47 mm MF-Millipore 0.05-µm pore disks (#VMWP04700; Merck Millipore) to float the samples on a 250-mL beaker of TE buffer (1 mM Tris at pH 8.0, 1 mM EDTA).

Genomic DNA samples were sequenced using single-end, 51-bp, bar-coded reads prepared and run using seqWell library prep kits (seqWell) and Illumina Next Seq 2000 (Illumina) following the manufacturer's instructions. Gene mutations in each strain were confirmed using the program Breseq to identify differences with the reference genome for SR108 [67]. For all strains, the original Illumina sequence reads were aligned to the SR108 reference genome and assembled using the program Bowtie 1.0.0 (94). All aligned reads were then characterized to determine the nucleotide frequency at each position. The number of sequences per kilobase was determined and plotted using a custom Python script. To prevent sequencing bias caused by the purification or sequencing, the copy number for each strain was normalized to a stationary-phase culture of SR108. Plots represent these relative copy number values at each genomic location in 1-kb bins and depict the replication profile of each strain. The 30kb (30bin) floating average was determined at each point and plotted as a solid line.

Results

Similar to BRCA2, RecF, but not RecBCD, maintains the DNA at replication forks disrupted by UV-induced DNA damage

In *E. coli*, the ability to protect replication forks following disruption by UV-induced DNA damage can be examined by following the fate of [^{3}H]pulse-labeled nascent DNA made just prior to UV-irradiation. To this end, [^{3}H]thymidine was added to growing cultures for 10 seconds before the culture was transferred to nonradioactive medium and irradiated with 40 J/m^2 UV. The amount of ^{3}H remaining in the DNA was then followed over time. This treatment produces an average of 1 lesion every 6 kb [63], and, with irradiation time, would be estimated to label 10-100 kb of DNA prior to the disruption of replication at sites of UV damage [47]. Greater than 95% of the parental cells survive this treatment, and replication efficiently resumes at a time that correlates and depends on repair of the UV lesions [49]. Thus, we infer that the events observed following these treatment conditions reflect mechanisms by which cells process, recover, and survive UV damage.

In UV-irradiated wild-type cells, some limited degradation of the nascent DNA occurs at times prior to the recovery of replication (Fig. 1A&B). In contrast, the degradation is much more extensive in *recF* mutants, continuing until approximately half of the nascent DNA has been degraded. By isolating the nascent DNA, the use of strand-specific probes has shown that the degradation in these mutants is predominantly targeted to the nascent lagging strand [51]. The extensive degradation in *recF* mutants correlates with a failure to restore DNA synthesis and cell death [47]. By contrast, the nascent DNA is not extensively degraded in mutants lacking RecBCD, despite the similar UV hypersensitivity in these cultures (Figure S1). Consistent with this, *recBCD* mutants resume replication following UV irradiation with kinetics that are similar to wild-type cells [47, 68, 69].

The failure to maintain or protect the nascent DNA in *recF* mutants can also be visualized by examining the structural intermediates arising at disrupted replication forks on plasmids such as pBR322 using two-dimensional (2-D) agarose gel analysis [49, 70]. Cells containing the plasmid pBR322 were grown and UV-irradiated as before, and the genomic DNA was then purified, digested with PvuII (which cuts the plasmid proximal to its unidirectional origin of replication), and analyzed by 2-D agarose gels. In this approach, nonreplicating plasmids migrate as linear 4.4-kb fragments, whereas replicating fragments, which form Y-shaped structures, migrate slower due to their nonlinear shape and larger size. The replicating fragments appear as an arc, extending out from the linear fragment (Fig. 1C). In wild-type cells, the arrested replication fork undergoes a transient reversal after UV irradiation which is protected and maintained by RecF, -O, and -R proteins until a time that correlates with lesion repair by nucleotide excision repair and the resumption of replication. The extrusion of the nascent DNA converts the three-arm, replication fork structure into a four-arm intermediate that further retards mobility in the gel and migrates in a cone region above the replication arc in the 2-D gel [71] (Fig. 1D). Consistent with this interpretation, in UV-irradiated *recF* mutants, the nascent DNA degrades, and regressed, cone-region intermediates are not observed. In contrast, the nascent DNA remains protected in *recBC* mutants and cone region intermediates appear similar to that seen in wild-type cells. Taken together, the results demonstrate that when replication is disrupted by UV-induced damage, RecF protects the nascent DNA from degradation, in a manner that resembles BRCA2 [3, 4], whereas RecBCD does not appear to process or access this substrate.

It is interesting to note that while both *recF* and *recBCD* mutants are sensitive to UV exposure, only *recF* shows an effect on processing of UV-arrested replication forks. Previous

studies have shown that following UV irradiation, replication continues to initiate from the origin in *recBCD* mutants, despite their inability to complete the ongoing rounds of replication [72]. This leads to large chromosomal imbalances centered around the origin, which may be responsible for the hypersensitivity observed in these mutants.

RecQ-RecJ (WRN-DNA2 homolog), but not SbcC-SbcD (MRE11-RAD50 homolog), process the DNA at UV-disrupted replication forks

In humans cells, both WRN-DNA2 and MRE11-RAD50 have been shown to partially degrade the nascent DNA of replication forks following hydroxyurea treatment [13, 14, 16, 73, 74].

In *E. coli*, the WRN and DNA2 homologs, RecQ and RecJ similarly degrade the nascent DNA following the disruption of replication by UV-induced damage [49–51, 75]. Consistent with their polarities, this degradation has been shown to predominantly occur on the nascent lagging strand. However, whether the SbcC-SbcD structure-specific nuclease can access or degrade these disrupted replication forks has not been examined.

To examine this question, we repeated the assay described above in *recF* mutants that lacked these respective enzymes. As shown in Figure 2A and consistent with previous observations, in the absence of either RecQ or RecJ, the nascent strand degradation at disrupted replication forks was eliminated during the initial time period prior to when replication normally resumes. The amount of degradation was also diminished throughout the 200 minute period of the assay, relative to that in the *recF* background alone. We infer that the degradation occurring during this later period likely represents aberrant processes that arise when replication fails to recover and cell lethality ensues. Two hundred minutes represents a duration of several cell cycles under these growth conditions, and greater than 99% of cells in these *recF* populations fail to resume replication or survive. When we examined mutants lacking SbcC and SbcD, the nascent DNA degradation occurred with the same kinetics and extent as in the *recF* background alone.

The lack of processing by SbcC-SbcD can also be observed by 2-D agarose gel analysis. Whereas in *recF* mutants, the regressed, nascent strand arm of the replication intermediate remains unprotected and degrades, it is restored following inactivation of RecJ or RecQ, which are responsible for its degradation (Figure 2B). However, inactivation of SbcC-SbcD was unable to restore this regressed intermediate in the *recF* mutants.

It remains possible that processing by SbcC-SbcD requires RecF to be recruited to the disrupted fork. To address this possibility, we also examined these mutants in an otherwise wild-type background. As shown in Figure 3, the limited degradation that occurs in wild-type cells did not occur in the absence of either RecJ or RecQ. However, the nascent degradation still occurred in the absence of SbcC-SbcD. These observations demonstrate that RecJ and RecQ partially degrade the nascent DNA at UV-disrupted replication forks, while RecF functions to limit the extent of degradation occurring. In contrast, *E. coli*'s SbcC-SbcD nuclease complex does not access or degrade the nascent DNA of disrupted forks either in the presence or absence of RecF protection. Taken together, these results indicate that SbcC-SbcD does not process or have access to replication forks disrupted by UV-induced damage.

RecBCD, but not RecF, protects DNA at sites where replication forks converge.

Recent work has demonstrated that completing DNA replication, a process in which two replisomes converge, is a highly-regulated process that requires unique enzymatic processing and is critical to maintain genome stability [76]. The completion of DNA replication can be challenging to study in human cells, in part because thousands of origins initiate with varying efficiencies and timing, making the loci where forks converge on the chromosomes highly variable between cells and cell cycles. However in *E. coli*, completion occurs once per cell cycle at a point opposite to its bidirectional origin of replication. The ability to complete replication can be observed by profiling the copy number of sequences around the genome in replicating cultures. In this technique, purified genomic DNA from replicating cultures is fragmented and then sequenced by high throughout sequencing. The replication profile can then be determined by counting the number of sequences that align to each region of the chromosome (Fig. 4A). In wild-type cultures, sequences surrounding the bidirectional origin are observed at higher frequencies because they replicate earlier than chromosome regions further removed from the origin. The a given sequence's frequency decreases inversely with its distance from the origin until reaching the region where replication forks converge where the completion of replication occurs (Fig. 4B). In the absence of *recF*, the profile of replication resembles that of wild-type cells. However, in *recBCD* mutants, a dramatic loss of sequences is observed in the region where replication forks converge (Fig. 4B). Considering that more than one half of sequence reads in a population must correspond to the parental DNA strands, the observed two-fold reduction in copy number of sequences in this region implies that most cells in the *recBCD* population are unable to maintain this region of the chromosome. The inability to maintain the chromosome in this region likely accounts for the impaired growth and low viability of these strains. Thus, whereas RecF protects the nascent DNA at replication forks disrupted by UV, RecBCD is specifically required to protect and prevent the degradation of DNA at sites where replication forks converge.

SbcC-SbcD, but not RecQ-RecJ, processes the DNA where replication forks converge

Recent work characterizing the completion of replication has shown that mutants lacking SbcC-SbcD along with Exo I of *E. coli* are susceptible to amplifications and genetic instability at loci where replication forks converge [60, 61, 76]. To determine if SbcC-SbcD is responsible for the degradation of DNA at these loci, we repeated the analysis above in *recBCD* mutants that also lacked the SbcC-SbcD nuclease. As shown in Figure 5, inactivation of SbcC-SbcD reduced the exonucleolytic degradation that occurred in the absence of RecBCD protection. In contrast, the amount and extent of degradation remained unchanged upon inactivation of the RecJ nuclease in the *recBCD* mutant, indicating that this enzyme does not access or process this substrate *in vivo*. Taken together, the observations demonstrate that the structure-specific nuclease, SbcC-SbcD, but not RecJ, processes the DNA at loci where replication forks converge.

Discussion

The results presented here indicate that whereas RecJ and RecQ process DNA at replication forks disrupted by UV photoproducts, SbcC and SbcD process DNA at sites where replication forks converge. While these proteins may have other cellular targets, the assays reveal that RecJ-RecQ and SbcC-SbcD each process unique replication fork DNA substrates and

operate at distinct loci on the genome. This seems likely to be true for the majority of situations when phenotypic redundancies between proteins are observed, given a lack of evolutionary pressure to maintain truly redundant gene products in a genome. While proteins may appear redundant with respect to a particular molecular or genetic phenotype, they likely have unique cellular functions which the assay employed cannot reveal.

The human homologs of RecQ-RecJ and SbcC-SbcD, WRN-DNA2 and MRE11-RAD50, have both been shown to process or partially degrade DNA at replication forks stalled by treatment with hydroxyurea [3, 13, 14]. Yet mutations in these gene products each give rise to distinct cancers, mutational signatures, and genetic instabilities, making it clear that they have distinct cellular functions. Given the high level of conservation in the mechanistic process of replication and repair, it is worth considering whether these various proteins associated with human cancers are conserved, or partially conserved, with their bacterial counterparts.

In the case of BRCA2, the bacterial RecF, RecO, and RecR proteins appear to play a functionally homologous role in protecting disrupted replication forks as well as limiting or regulating the amount of nascent DNA that is degraded, despite a lack of sequence or structural similarity (Figure 6). However, other human enzymes have also been reported to promote stabilization and protection following stalls induced by hydroxyurea or disruption by cisplatin, including FANCD2, ABRO1, BOD1L, VHL, and FANCA, suggesting these as other potential candidates for RecF-O-R homologs [9–12]. Perhaps structural similarities between BCRA2 or other candidates will be revealed as we learn more about the specific conditions and substrates which manifest their protection of replication fork DNA.

WRN and DNA2 appear to play a similar role in processing the nascent DNA at disrupted replication forks as their bacterial counterparts, RecQ and RecJ (Figure 6). WRN is a RecQ-family helicase that shares partial sequence and structural similarity with RecQ [77]. Although, DNA2 and RecJ do not contain any clear sequence homology, they do exhibit homology at a functional level. Both DNA2 and RecJ encode 5’-exonucleases that act in concert with RECQ-family helicases, and are stimulated by single-strand binding protein [13, 16, 18, 51, 53, 54]. Several studies have also reported that BLM, another RecQ-family helicase which causes Bloom’s syndrome and cancer predisposition when mutated, also interacts and functions with either DNA2, or EXO5, a 5’ exonuclease, to process replication forks in a manner that appears similar to WRN-DNA2, arguing that other RecQ-RecJ homologs may also operate at the replication fork under alternative, or as yet, unidentified conditions [16, 73, 78].

The case of MRE11-RAD50 and the question of its functional homology with SbcC-SbcD is more complex. Both proteins contain significant sequence and structural conservations with their bacterial counterparts. However, in this work, we show that SbcC-SbcD does not process nascent DNA at disrupted replication forks, but instead processes DNA at sites where replication forks converge. Thus, one possibility is that despite their sequence and structural conservation, *E. coli* SbcC-SbcD are not functionally conserved with their human counterparts.

The alternative possibility, worth considering, is that these proteins are functionally conserved, and that human MRE11-RAD50 may not normally process DNA at stalled elongating replication forks, but instead process other loci, such as those where replication forks converge (Figure 7). The most common assay for monitoring replication fork processing in human cells utilizes sequential pulse-labeling of cultures with the thymidine analogs, IdU and then CldU, before treatment with hydroxyurea to stall replication. Genomic DNA is then purified from cells

at various time points and spread on glass slides, before the labeled strands are visualized using differently labeled fluorescent antibodies against each thymidine analog. Nascent DNA degradation is inferred from a reduction in the length of the second, CldU tract-length, relative to the first, IdU tract-length, over time following replication inhibition [3, 13].

Aspects of this assay suggest that other events, in addition to elongating replication forks, could also be captured during the labelling process. *E. coli*'s 4.5-megabase genome contains a single origin and completion site. This means that a 5-sec pulse-label of replication forks progressing ~10^3 bases per second encompasses primarily elongating replication forks [79]. In contrast, human cells contain thousands of origins and completion sites along their chromosomes, averaging ~100 kilobases between these two points, with considerable variance [80]. The two pulse-labels used in human cell assays often range from 20 to 90 minutes [3, 14, 30]. With an average fork speed of 50-100 bases per second, the labeled population would cover most, or even entire replicons, and encompass significant proportions of initiation and completion events, in addition to the elongation events [81]. Thus, although these assays typically attribute the degradation to stalled elongation complexes, it remains possible that the degradation occurs at other loci or substrates captured by this technique. This possibility increases when one considers that only a small fraction of the labeled DNA is typically observed to degrade within the subpopulation of the total labeled events [3, 14, 30].

There are other observations that suggest MRE11-RAD50 may be operating at sites other than hydroxyurea-stalled replication forks. Processing by WRN-DNA2 or BLM-EXO5 is detected in otherwise wild-type cells, and the processing is needed for the replication to resume normally [13, 20, 73]. Similarly, RecQ-RecJ processing is critical for replication to resume normally, and promote survival without mutagenesis [52]. In contrast, processing of hydroxyurea-stalled replication forks by MRE11-RAD50 is only observed in the absence of protection by BRCA2 [3, 29–31]. The presence of other fork protection proteins, including FANCJ, has also been reported to prevent MRE11-RAD50 from accessing stalled replication forks [82]. Also notable is that Mre11 mutants are not hypersensitive to hydroxyurea [83]. Taken together, the observations suggest that under normal conditions, MRE11-RAD50 may not process, or have access to these elongating forks and that they may process other substrates in the cell, such as convergent replication forks, that account for its essential role in embryonic development and the normal cell cycle progression.

Although, analogous intermediates of the completion reaction have not been clearly established in humans, potential candidates or homologs have been identified. hPif1, is a helicase, associated with cancer growth that shares homology with RecD [84, 85] (Figure 7). While hPif1 phenotypes are pleiotropic and its precise cellular role remains undefined, it has been suggested to have a replicational roles in a range of processes, including telomere maintenance, resolving G-quartets, and unwinding RNA-DNA hybrids [86–88]. However in yeast, the hPif1 homologs, Pif1 and Rrm3 are required to allow replication forks to converge, and give rise to under-replicated intermediates, similar to *recBCD* in *E. coli* [89–92].

E. coli has long served as a useful model to dissect the highly conserved processes of replication and repair in eukaryotes and mammals. This remains true and is sometimes under-utilized. With respect to MRE11 and RAD50, it is worth considering whether the genomic instabilities arising in cancers containing mutations in these genes results from a fundamental process such as completing replication. Many of the completion enzymes in *E. coli* are also

sensitive to agents that generate double-stand breaks, and it has been noted that many double-strand break repair intermediates resemble structures generated during the completion of replication [93, 94]. Considering that MRE11-RAD50 also exhibit these hypersensitivities, perhaps this may represent a form of damage where the enzyme complex is recruited to and required for fork processing. Both replication initiation and completion events have distinct features that can be identified and characterized in assays similar to those currently used to assess fork processing in human cells. While alternative assays, similar to the replication profiles used in *E. coli*, may also prove useful in addressing this question.

Data availability

Sequencing data presented in this paper has been deposited in the Sequence Read Archive (SRA), and is available at https://www.ncbi.nlm. nih.gov/sra/PRJNA1335257 (accession no. PRJNA1335257). All other data is contained in the manuscript and Supplementary Information.

Author Contributions

RLS and JC wrote the initial manuscript draft. RLS, PYH, CTC and JC contributed to the experimental design, data collection, analysis, manuscript editing, and revisions.

Conflict of Interest

The authors declare they have no conflicts of interest.

Acknowledgements

This work was supported by grants from the National Science Foundation (MCB1916625), the NIH National Institute of General Medical Sciences (R16GM14554), and the NIH National Institute of Environmental Health Sciences (R21ES034880).

References

[1] Chatterjee, N. and Walker, G.C. (2017) Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* **58**, 235-263.
[2] Couch, F.J., Nathanson, K.L. and Offit, K. (2014) Two decades after BRCA: setting paradigms in personalized cancer care and prevention. *Science* **343**, 1466-1470.
[3] Schlacher, K., Christ, N., Siaud, N., Egashira, A., Wu, H. and Jasin, M. (2011) Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. *Cell* **145**, 529-542.
[4] Schlacher, K., Wu, H. and Jasin, M. (2012) A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer Cell* **22**, 106-116.
[5] Shin, D.S., Pellegrini, L., Daniels, D.S., Yelent, B., Craig, L., Bates, D., Yu, D.S., Shivji, M.K., Hitomi, C., Arvai, A.S., Volkmann, N., Tsuruta, H., Blundell, T.L., Venkitaraman, A.R. and Tainer, J.A. (2003) Full-length archaeal Rad51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *EMBO J* **22**, 4566-4576.
[6] Sung, P. and Robberson, D.L. (1995) DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell* **82**, 453-461.
[7] Mason, J.M., Chan, Y.L., Weichselbaum, R.W. and Bishop, D.K. (2019) Non-enzymatic roles of human RAD51 at stalled replication forks. *Nat Commun* **10**, 4410.

[8] González-Prieto, R., Muñoz-Cabello, A.M., Cabello-Lobato, M.J. and Prado, F. (2013) Rad51 replication fork recruitment is required for DNA damage tolerance. *EMBO J* **32**, 1307-1321.
[9] Xu, S., Wu, X., Wu, L., Castillo, A., Liu, J., Atkinson, E., Paul, A., Su, D., Schlacher, K., Komatsu, Y., You, M.J. and Wang, B. (2017) Abro1 maintains genome stability and limits replication stress by protecting replication fork stability. *Genes Dev* **31**, 1469-1482.
[10] Higgs, M.R., Reynolds, J.J., Winczura, A., Blackford, A.N., Borel, V., Miller, E.S., Zlatanou, A., Nieminuszczy, J., Ryan, E.L., Davies, N.J., Stankovic, T., Boulton, S.J., Niedzwiedz, W. and Stewart, G.S. (2015) BOD1L Is Required to Suppress Deleterious Resection of Stressed Replication Forks. *Mol Cell* **59**, 462-477.
[11] Espana-Agusti, J., Warren, A., Chew, S.K., Adams, D.J. and Matakidou, A. (2017) Loss of PBRM1 rescues VHL dependent replication stress to promote renal carcinogenesis. *Nat Commun* **8**, 2026.
[12] Liu, W., Krishnamoorthy, A., Zhao, R. and Cortez, D. (2020) Two replication fork remodeling pathways generate nuclease substrates for distinct fork protection factors. *Sci Adv* **6**,
[13] Thangavel, S., Berti, M., Levikova, M., Pinto, C., Gomathinayagam, S., Vujanovic, M., Zellweger, R., Moore, H., Lee, E.H., Hendrickson, E.A., Cejka, P., Stewart, S., Lopes, M. and Vindigni, A. (2015) DNA2 drives processing and restart of reversed replication forks in human cells. *J Cell Biol* **208**, 545-562.
[14] Lemaçon, D., Jackson, J., Quinet, A., Brickner, J.R., Li, S., Yazinski, S., You, Z., Ira, G., Zou, L., Mosammaparast, N. and Vindigni, A. (2017) MRE11 and EXO1 nucleases degrade reversed forks and elicit MUS81-dependent fork rescue in BRCA2-deficient cells. *Nat Commun* **8**, 860.
[15] Budd, M.E., Choe, W.C. and Campbell, J.L. (1995) DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes. *J Biol Chem* **270**, 26766-26769.
[16] Pinto C, Kasaciunaite K and Seidel R, C.P. (2016) Human DNA2 possesses a cryptic DNA unwinding activity that functionally integrates with BLM or WRN helicases. *eLIFE* **5:e18574**, 1-24.
[17] Paull, T.T. and Gellert, M. (1999) Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev* **13**, 1276-1288.
[18] Brosh, R.M., Jr., Orren, D.K., Nehlin, J.O., Ravn, P.H., Kenny, M.K., Machwe, A. and Bohr, V.A. (1999) Functional and physical interaction between WRN helicase and human replication protein A. *J Biol Chem* **274**, 18341-18350.
[19] Datta, A., Biswas, K., Sommers, J.A., Thompson, H., Awate, S., Nicolae, C.M., Thakar, T., Moldovan, G.L., Shoemaker, R.H., Sharan, S.K. and Brosh, R.M. (2021) WRN helicase safeguards deprotected replication forks in BRCA2-mutated cancer cells. *Nat Commun* **12**, 6561.
[20] Pichierri, P., Franchitto, A., Mosesso, P. and Palitti, F. (2001) Werner's syndrome protein is required for correct recovery after replication arrest and DNA damage induced in S-phase of cell cycle. *Mol Biol Cell* **12**, 2412-2421.
[21] Epstein, C.J. and Motulsky, A.G. (1996) Werner syndrome: entering the helicase era. *Bioessays* **18**, 1025-1027.
[22] Mojumdar, A. (2020) Mutations in conserved functional domains of human RecQ helicases are associated with diseases and cancer: A review. *Biophys Chem* **265**, 106433.
[23] Folly-Kossi, H., Graves, J.D., Garan, L.A.W., Lin, F.T. and Lin, W.C. (2023) DNA2 Nuclease Inhibition Confers Synthetic Lethality in Cancers with Mutant p53 and Synergizes with PARP Inhibitors. *Cancer Res Commun* **3**, 2096-2112.

[24] Hopfner, K.P., Craig, L., Moncalian, G., Zinkel, R.A., Usui, T., Owen, B.A., Karcher, A., Henderson, B., Bodmer, J.L., McMurray, C.T., Carney, J.P., Petrini, J.H. and Tainer, J.A. (2002) The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. *Nature* **418**, 562-566.
[25] Lobachev, K.S., Gordenin, D.A. and Resnick, M.A. (2002) The Mre11 complex is required for repair of hairpin-capped double-strand breaks and prevention of chromosome rearrangements. *Cell* **108**, 183-193.
[26] Trujillo, K.M. and Sung, P. (2001) DNA structure-specific nuclease activities in the Saccharomyces cerevisiae Rad50*Mre11 complex. *J Biol Chem* **276**, 35458-35464.
[27] Paull, T.T. and Gellert, M. (1998) The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell* **1**, 969-979.
[28] Trujillo, K.M., Yuan, S.S., Lee, E.Y. and Sung, P. (1998) Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. *J Biol Chem* **273**, 21447-21450.
[29] Taglialatela, A., Alvarez, S., Leuzzi, G., Sannino, V., Ranjha, L., Huang, J.W., Madubata, C., Anand, R., Levy, B., Rabadan, R., Cejka, P., Costanzo, V. and Ciccia, A. (2017) Restoration of Replication Fork Stability in BRCA1- and BRCA2-Deficient Cells by Inactivation of SNF2-Family Fork Remodelers. *Mol Cell* **68**, 414-430.e8.
[30] Ray Chaudhuri, A., Callen, E., Ding, X., Gogola, E., Duarte, A.A., Lee, J.E., Wong, N., Lafarga, V., Calvo, J.A., Panzarino, N.J., John, S., Day, A., Crespo, A.V., Shen, B., Starnes, L.M., de Ruiter, J.R., Daniel, J.A., Konstantinopoulos, P.A., Cortez, D., Cantor, S.B., Fernandez-Capetillo, O., Ge, K., Jonkers, J., Rottenberg, S., Sharan, S.K. and Nussenzweig, A. (2016) Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* **535**, 382-387.
[31] Ying, S., Hamdy, F.C. and Helleday, T. (2012) Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Res* **72**, 2814-2821.
[32] Ajimura, M., Leem, S.H. and Ogawa, H. (1993) Identification of new genes required for meiotic recombination in Saccharomyces cerevisiae. *Genetics* **133**, 51-66.
[33] Luo, G., Yao, M.S., Bender, C.F., Mills, M., Bladl, A.R., Bradley, A. and Petrini, J.H. (1999) Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A* **96**, 7376-7381.
[34] Petrini, J.H., Walsh, M.E., DiMare, C., Chen, X.N., Korenberg, J.R. and Weaver, D.T. (1995) Isolation and characterization of the human MRE11 homologue. *Genomics* **29**, 80-86.
[35] Deng, S.K., Yin, Y., Petes, T.D. and Symington, L.S. (2015) Mre11-Sae2 and RPA Collaborate to Prevent Palindromic Gene Amplification. *Mol Cell* **60**, 500-508.
[36] Lengsfeld, B.M., Rattray, A.J., Bhaskara, V., Ghirlando, R. and Paull, T.T. (2007) Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. *Mol Cell* **28**, 638-651.
[37] Rattray, A.J., Shafer, B.K., Neelam, B. and Strathern, J.N. (2005) A mechanism of palindromic gene amplification in Saccharomyces cerevisiae. *Genes Dev* **19**, 1390-1399.
[38] Waltes, R., Kalb, R., Gatei, M., Kijas, A.W., Stumm, M., Sobeck, A., Wieland, B., Varon, R., Lerenthal, Y., Lavin, M.F., Schindler, D. and Dörk, T. (2009) Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. *Am J Hum Genet* **84**, 605-616.
[39] Stewart, G.S., Maser, R.S., Stankovic, T., Bressan, D.A., Kaplan, M.I., Jaspers, N.G., Raams, A., Byrd, P.J., Petrini, J.H. and Taylor, A.M. (1999) The DNA double-strand break

574 repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell*
575 **99**, 577-587.
576 [40] Xiao, Y. and Weaver, D.T. (1997) Conditional gene targeted deletion by Cre recombinase
577 demonstrates the requirement for the double-strand break repair Mre11 protein in murine
578 embryonic stem cells. *Nucleic Acids Res* **25**, 2985-2991.
579 [41] Rahman, S., Canny, M.D., Buschmann, T.A. and Latham, M.P. (2020) A Survey of
580 Reported Disease-Related Mutations in the MRE11-RAD50-NBS1 Complex. *Cells* **9**,
581 [42] Zhou, T. and Zhang, J. (2025) Therapeutic advances and application of PARP inhibitors in
582 breast cancer. *Transl Oncol* **57**, 102410.
583 [43] Hao, S., Liu, Z., Lenz, H.J., Yu, J. and Zhang, L. (2025) Werner helicase as a therapeutic
584 target in mismatch repair deficient colorectal cancer. *DNA Repair (Amst)* **149**, 103831.
585 [44] Webb, B.L., Cox, M.M. and Inman, R.B. (1997) Recombinational DNA repair: the RecF
586 and RecR proteins limit the extension of RecA filaments beyond single-strand DNA gaps. *Cell*
587 **91**, 347-356.
588 [45] Bork, J.M., Cox, M.M. and Inman, R.B. (2001) The RecOR proteins modulate RecA protein
589 function at 5' ends of single- stranded DNA. *Embo J* **20**, 7313-22.
590 [46] Umezu, K. and Kolodner, R.D. (1994) Protein interactions in genetic recombination in
591 Escherichia coli. Interactions involving RecO and RecR overcome the inhibition of RecA by
592 single-stranded DNA-binding protein. *J Biol Chem* **269**, 30005-30013.
593 [47] Courcelle, J., Carswell-Crumpton, C. and Hanawalt, P.C. (1997) recF and recR are required
594 for the resumption of replication at DNA replication forks in Escherichia coli. *Proc Natl Acad*
595 *Sci U S A* **94**, 3714-9.
596 [48] Courcelle, J., Crowley, D.J. and Hanawalt, P.C. (1999) Recovery of DNA replication in
597 UV-irradiated Escherichia coli requires both excision repair and recF protein function. *J*
598 *Bacteriol* **181**, 916-22.
599 [49] Courcelle, J., Donaldson, J.R., Chow, K.H. and Courcelle, C.T. (2003) DNA Damage-
600 Induced Replication Fork Regression and Processing in Escherichia coli. *Science* **299**, 1064-
601 1067.
602 [50] Chow, K.H. and Courcelle, J. (2004) RecO Acts with RecF and RecR to Protect and
603 Maintain Replication Forks Blocked by UV-induced DNA Damage in Escherichia coli. *J Biol*
604 *Chem* **279**, 3492-3496.
605 [51] Courcelle, J. and Hanawalt, P.C. (1999) RecQ and RecJ process blocked replication forks
606 prior to the resumption of replication in UV-irradiated Escherichia coli. *Mol Gen Genet* **262**,
607 543-51.
608 [52] Courcelle, C.T., Chow, K.H., Casey, A. and Courcelle, J. (2006) Nascent DNA processing
609 by RecJ favors lesion repair over translesion synthesis at arrested replication forks in Escherichia
610 coli. *Proc Natl Acad Sci U S A* **103**, 9154-9159.
611 [53] Han, E.S., Cooper, D.L., Persky, N.S., Sutera, V.A., Whitaker, R.D., Montello, M.L. and
612 Lovett, S.T. (2006) RecJ exonuclease: substrates, products and interaction with SSB. *Nucleic*
613 *Acids Res* **34**, 1084-1091.
614 [54] Shereda, R.D., Reiter, N.J., Butcher, S.E. and Keck, J.L. (2009) Identification of the SSB
615 binding site on E. coli RecQ reveals a conserved surface for binding SSB's C terminus. *J Mol*
616 *Biol* **386**, 612-625.
617 [55] Rudolph, C.J., Upton, A.L. and Lloyd, R.G. (2008) Maintaining replication fork integrity in
618 UV-irradiated Escherichia coli cells. *DNA Repair (Amst)* **7**, 1589-1602.

[56] Courcelle, C.T., Belle, J.J. and Courcelle, J. (2005) Nucleotide excision repair or polymerase V-mediated lesion bypass can act to restore UV-arrested replication forks in Escherichia coli. *J Bacteriol* **187**, 6953-6961.
[57] Lloyd, R.G. and Buckman, C. (1985) Identification and genetic analysis of sbcC mutations in commonly used recBC sbcB strains of Escherichia coli K-12. *J Bacteriol* **164**, 836-44.
[58] Gibson, F.P., Leach, D.R. and Lloyd, R.G. (1992) Identification of sbcD mutations as cosuppressors of recBC that allow propagation of DNA palindromes in Escherichia coli K-12. *J Bacteriol* **174**, 1222-8.
[59] Chalker, A.F., Leach, D.R. and Lloyd, R.G. (1988) Escherichia coli sbcC mutants permit stable propagation of DNA replicons containing a long palindrome. *Gene* **71**, 201-205.
[60] Rudolph, C.J., Upton, A.L., Stockum, A., Nieduszynski, C.A. and Lloyd, R.G. (2013) Avoiding chromosome pathology when replication forks collide. *Nature* **500**, 608-611.
[61] Wendel, B.M., Cole, J.M., Courcelle, C.T. and Courcelle, J. (2018) SbcC-SbcD and ExoI process convergent forks to complete chromosome replication. *Proc Natl Acad Sci U S A* **115**, 349-354.
[62] Hamilton, N.A., Wendel, B.M., Weber, E.A., Courcelle, C.T. and Courcelle, J. (2019) RecBCD, SbcCD and ExoI process a substrate created by convergent replisomes to complete DNA replication. *Mol Microbiol* **111**, 1638-1651.
[63] Mellon, I. and Hanawalt, P.C. (1989) Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature* **342**, 95-98.
[64] Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L. and Mori, H. (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006.0008.
[65] Murphy, K.C., Campellone, K.G. and Poteete, A.R. (2000) PCR-mediated gene replacement in Escherichia coli. *Gene* **246**, 321-330.
[66] Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95-113.
[67] Barrick, J.E., Colburn, G., Deatherage, D.E., Traverse, C.C., Strand, M.D., Borges, J.J., Knoester, D.B., Reba, A. and Meyer, A.G. (2014) Identifying structural variation in haploid microbial genomes from short-read resequencing data using breseq. *BMC Genomics* **15**, 1039.
[68] Courcelle, J. and Hanawalt, P.C. (2001) Participation of recombination proteins in rescue of arrested replication forks in UV-irradiated Escherichia coli need not involve recombination. *Proc Natl Acad Sci U S A* **98**, 8196-202.
[69] Al-Hadid, Q., Ona, K., Courcelle, C.T. and Courcelle, J. (2008) RecA433 cells are defective in recF-mediated processing of disrupted replication forks but retain recBCD-mediated functions. *Mutat Res* **645**, 19-26.
[70] Friedman, K.L. and Brewer, B.J. (1995) Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol* **262**, 613-627.
[71] Donaldson, J.R., Courcelle, C.T. and Courcelle, J. (2006) RuvABC is required to resolve holliday junctions that accumulate following replication on damaged templates in Escherichia coli. *J Biol Chem* **281**, 28811-28821.
[72] Wendel, B.M., Hollingsworth, S., Courcelle, C.T. and Courcelle, J. (2021) UV-induced DNA damage disrupts the coordination between replication initiation, elongation and completion. *Genes Cells* **26**, 94-108.

[73] Hambarde, S., Tsai, C.L., Pandita, R.K., Bacolla, A., Maitra, A., Charaka, V., Hunt, C.R., Kumar, R., Limbo, O., Le Meur, R., Chazin, W.J., Tsutakawa, S.E., Russell, P., Schlacher, K., Pandita, T.K. and Tainer, J.A. (2021) EXO5-DNA structure and BLM interactions direct DNA resection critical for ATR-dependent replication restart. *Mol Cell* **81**, 2989-3006.e9.
[74] Bhat, K.P., Krishnamoorthy, A., Dungrawala, H., Garcin, E.B., Modesti, M. and Cortez, D. (2018) RADX Modulates RAD51 Activity to Control Replication Fork Protection. *Cell Rep* **24**, 538-545.
[75] Chow, K.H. and Courcelle, J. (2007) RecBCD and RecJ/RecQ initiate DNA degradation on distinct substrates in UV-irradiated Escherichia coli. *Radiat Res* **168**, 499-506.
[76] Wendel, B.M., Courcelle, C.T. and Courcelle, J. (2014) Completion of DNA replication in Escherichia coli. *Proc Natl Acad Sci U S A* **111**, 16454-16459.
[77] Gray, M.D., Shen, J.C., Kamath-Loeb, A.S., Blank, A., Sopher, B.L., Martin, G.M., Oshima, J. and Loeb, L.A. (1997) The Werner syndrome protein is a DNA helicase. *Nat Genet* **17**, 100-103.
[78] Seppa, I.M., Ceppi, I., Tennakoon, M., Reginato, G., Jackson, J., Rouault, C.D., Agashe, S., Sviderskiy, V.O., Limbu, M., Lantelme, E., Meroni, A., Braunshier, S., Borrello, D., Verma, P., Cejka, P. and Vindigni, A. (2025) MRN-CtIP, EXO1, and DNA2-WRN/BLM act bidirectionally to process DNA gaps in PARPi-treated cells without strand cleavage. *Genes Dev* **39**, 582-602.
[79] Marians, K.J. (1992) Prokaryotic DNA replication. *Annu Rev Biochem* **61**, 673-719.
[80] Tian, M., Wang, Z., Su, Z., Shibata, E., Shibata, Y., Dutta, A. and Zang, C. (2024) Integrative analysis of DNA replication origins and ORC-/MCM-binding sites in human cells reveals a lack of overlap. *Elife* **12**,
[81] Painter, R.B. and Schaefer, A.W. (1971) Variation in the rate of DNA chain growth through the S phase in HeLa cells. *J Mol Biol* **58**, 289-295.
[82] Suhasini, A.N., Sommers, J.A., Muniandy, P.A., Coulombe, Y., Cantor, S.B., Masson, J.Y., Seidman, M.M. and Brosh, R.M. (2013) Fanconi anemia group J helicase and MRE11 nuclease interact to facilitate the DNA damage response. *Mol Cell Biol* **33**, 2212-2227.
[83] Lam, A.F., Krogh, B.O. and Symington, L.S. (2008) Unique and overlapping functions of the Exo1, Mre11 and Pso2 nucleases in DNA repair. *DNA Repair (Amst)* **7**, 655-662.
[84] Zhang, D.H., Zhou, B., Huang, Y., Xu, L.X. and Zhou, J.Q. (2006) The human Pif1 helicase, a potential Escherichia coli RecD homologue, inhibits telomerase activity. *Nucleic Acids Res* **34**, 1393-1404.
[85] Gagou, M.E., Ganesh, A., Thompson, R., Phear, G., Sanders, C. and Meuth, M. (2011) Suppression of apoptosis by PIF1 helicase in human tumor cells. *Cancer Res* **71**, 4998-5008.
[86] Schulz, V.P. and Zakian, V.A. (1994) The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell* **76**, 145-155.
[87] Sanders, C.M. (2010) Human Pif1 helicase is a G-quadruplex DNA-binding protein with G-quadruplex DNA-unwinding activity. *Biochem J* **430**, 119-128.
[88] Tran, P.L.T., Pohl, T.J., Chen, C.F., Chan, A., Pott, S. and Zakian, V.A. (2017) PIF1 family DNA helicases suppress R-loop mediated genome instability at tRNA genes. *Nat Commun* **8**, 15025.
[89] Ivessa, A.S., Zhou, J.Q. and Zakian, V.A. (2000) The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA. *Cell* **100**, 479-489.

[90] Deegan, T.D., Baxter, J., Ortiz Bazán, M.Á., Yeeles, J.T.P. and Labib, K.P.M. (2019) Pif1-Family Helicases Support Fork Convergence during DNA Replication Termination in Eukaryotes. *Mol Cell* **74**, 231-244.e9.
[91] Steinacher, R., Osman, F., Dalgaard, J.Z., Lorenz, A. and Whitby, M.C. (2012) The DNA helicase Pfh1 promotes fork merging at replication termination sites to ensure genome stability. *Genes Dev* **26**, 594-602.
[92] Su, N., Byrd, A.K., Bharath, S.R., Yang, O., Jia, Y., Tang, X., Ha, T., Raney, K.D. and Song, H. (2019) Structural basis for DNA unwinding at forked dsDNA by two coordinating Pif1 helicases. *Nat Commun* **10**, 5375.
[93] Courcelle, J., Wendel, B.M., Livingstone, D.D. and Courcelle, C.T. (2015) RecBCD is required to complete chromosomal replication: Implications for double-strand break frequencies and repair mechanisms. *DNA Repair (Amst)* **32**, 86-95.
[94] Wendel, B.M., Hernandez, A.J., Courcelle, C.T. and Courcelle, J. (2021) Ligase A and RNase HI Participate in Completing Replication on the Chromosome in Escherichia coli. *DNA* **1**, 13-25.
[95] Jeiranian, H.A., Schalow, B.J., Courcelle, C.T. and Courcelle, J. (2013) Fate of the replisome following arrest by UV-induced DNA damage in Escherichia coli. *Proc Natl Acad Sci U S A* **110**, 11421-11426.
[96] Hamilton, N.A., Jehru, A.E., Samples, W.N., Wendel, B.M., Mokhtari, P.D., Courcelle, C.T. and Courcelle, J. (2023) chi sequences switch the RecBCD helicase-nuclease complex from degradative to replicative modes during the completion of DNA replication. *J Biol Chem* 103013.
[97] Midgley-Smith, S.L., Dimude, J.U. and Rudolph, C.J. (2019) A role for 3' exonucleases at the final stages of chromosome duplication in Escherichia coli. *Nucleic Acids Res* **47**, 1847-1860.
[98] Sharples, G.J. and Leach, D.R. (1995) Structural and functional similarities between the SbcCD proteins of Escherichia coli and the RAD50 and MRE11 (RAD32) recombination and repair proteins of yeast. *Mol Microbiol* **17**, 1215-1217.

Figure Legends

Figure 1. RecFOR, but not RecBCD, protects the nascent DNA at replication forks disrupted by UV-induced damage. (A) *The strategy for [^{3}H]-labelling the nascent DNA and total genomic DNA is diagrammed.* A replicating circular *E. coli* genome is shown in each panel with the [^{3}H]-labeled DNA shown in red. (B) *RecF, but not RecBCD, is required to protect the nascent DNA from degradation.* The fraction of the radioactivity remaining in the DNA after UV irradiation is plotted over time. The loss of nascent DNA (solid symbols, left panel) and genomic DNA (open symbols, right panel) is shown for the parental strain (squares), *recF* (triangles), and *recBCD* (circles). Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean. C) *Diagram depicting the migration pattern of replication intermediates following Two-Dimensional (2-D) agarose gel analysis.* The position of i) nonreplicating fragments, ii) replicating fragments, and iii) disrupted replication fork intermediates is shown. D) *RecF, but not RecBCD, is required to maintain the integrity of the disrupted replication fork intermediates.* Cells containing the plasmid pBR322 were UV-irradiated and analyzed by 2-D gels at the times indicated. In *recF* mutants, the nascent DNA is degraded after UV irradiation, and the replication fork intermediates do not accumulate.

Figure 2. RecJ and RecQ, but not SbcC-SbcD, degrade the nascent DNA at replication forks disrupted by UV-induced damage in the absence of RecF protection. (A) Inactivation of *RecJ or RecQ, but not SbcC, prevents the nascent DNA* d*egradation in UV-irradiated recF mutants.* Cultures were treated and analyzed as in Fig 1A and B. The loss of nascent DNA is plotted over time for *recF* (squares), *recFrecJ* (triangles), *recFrecQ* (inverted triangles), and *recFsbcC* (diamonds) mutants. Data for *recF* mutants is reproduced from Figure 1 and shown for comparison. Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean. (B) Inactivation of *RecJ or RecQ, but not SbcC, partially restores the disrupted replication fork intermediates in recF mutants.* Cultures were treated and analyzed as in Fig 1C and D. Southern blots of the migration pattern of replicating pBR322 fragments are shown following electrophoresis in 2D agarose gels for *recF*, *recFrecJ*, *recFrecQ*, and *recFsbcC* mutants after UV irradiation at the times indicated.

Figure 3. RecJ and RecQ, but not SbcC, degrades the nascent DNA at replication forks disrupted by UV-induced damage in wild-type cells. Inactivation of *RecJ or RecQ, but not SbcC, prevents the nascent DNA* d*egradation in UV-irradiated cells.* Cultures were treated and analyzed as in Fig 1A and B. The loss of nascent DNA is plotted over time for wild-type (squares), *recJ* (triangles), *recQ* (inverted triangles), and *sbcC* (diamonds) mutants. Data for wild-type cells is reproduced from Figure 1 and shown for comparison. Plots represent the average of at least three independent experiments. Error bars represent the standard error of the mean.

Figure 4. RecBCD, but not RecF, protects the DNA at replication forks at loci where the replisomes converge. (A) *Diagram depicting the strategy of profiling replication across the E. coli genome*. (B) *Inactivation of recBCD, but not recF, leads to extensive degradation at loci where replication forks converge*. Genomic DNA from replicating cultures was purified, fragmented, and profiled using high-throughput sequencing. Normalized sequence read frequencies are plotted relative to genome position. The line represents the 30-kb floating average. The origin and terminus region where replication completes is indicated.

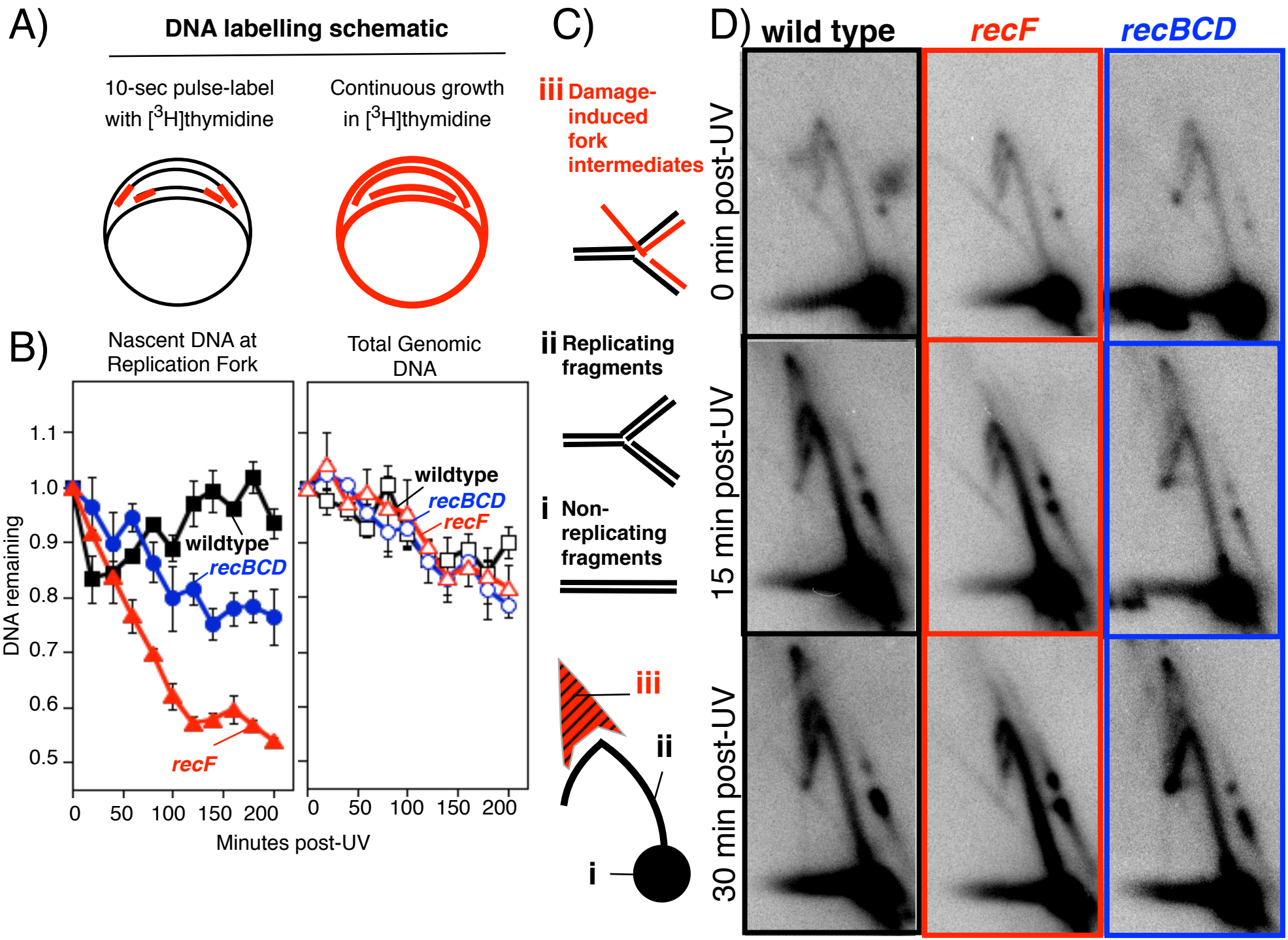
Figure 5. SbcC-SbcD, but not RecJ, processes the DNA at replication forks at loci where the replisomes converge. Inactivation of *sbcC-sbcD*, but not *recJ*, reduces the amount of degradation in *recBCD* mutants at loci where replication forks converge. Genomic DNA was isolated and analyzed as in Fig 4. Replication profiles for *recBCD*, *recBCDsbcCD*, and *recBCDrecJ* are plotted relative to genome position. Data for *recBCD* mutants is reproduced from Fig 4 and shown for comparison.

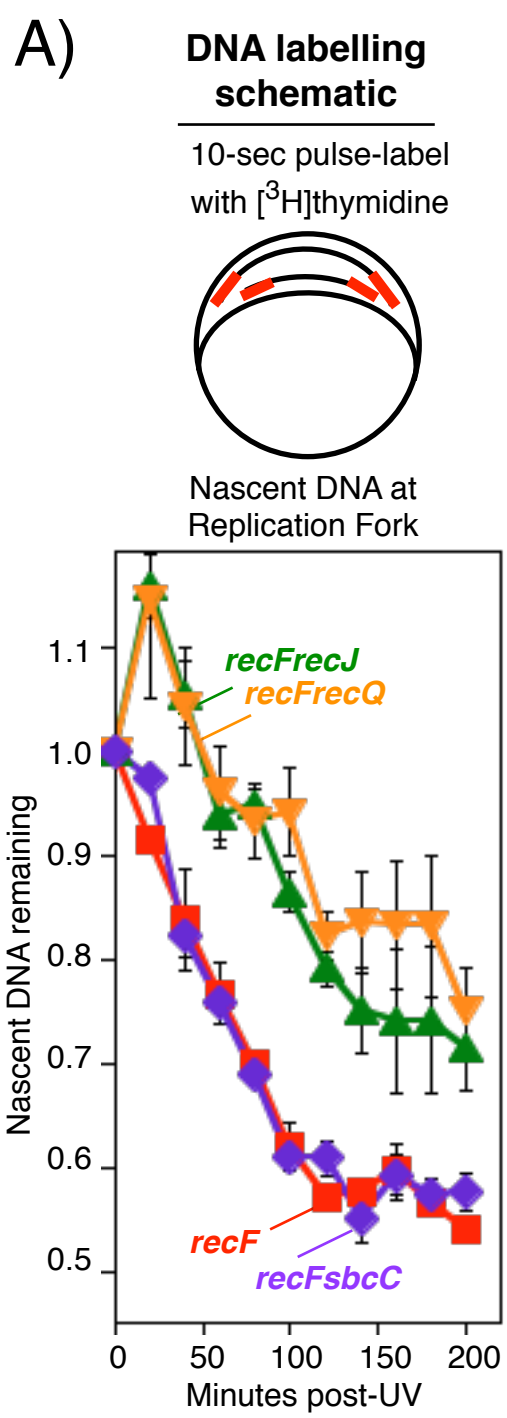
Figure 6. Comparing Models of *E. coli* and Human Activities Associated with Restoring Replication Following Disruption by DNA damage. Purple, Replisome Proteins; Green, Helicase-Primase; Green Line, nascent DNA; Black Lines, Parental DNA; ^, UV-induced lesion; Red, *E. coli* Proteins; Blue, human proteins. (*E. coli* model derived in part from data presented here and in [47–51, 95], Human model derived in part from data presented in [3, 3–5, 7, 8, 13, 14]).

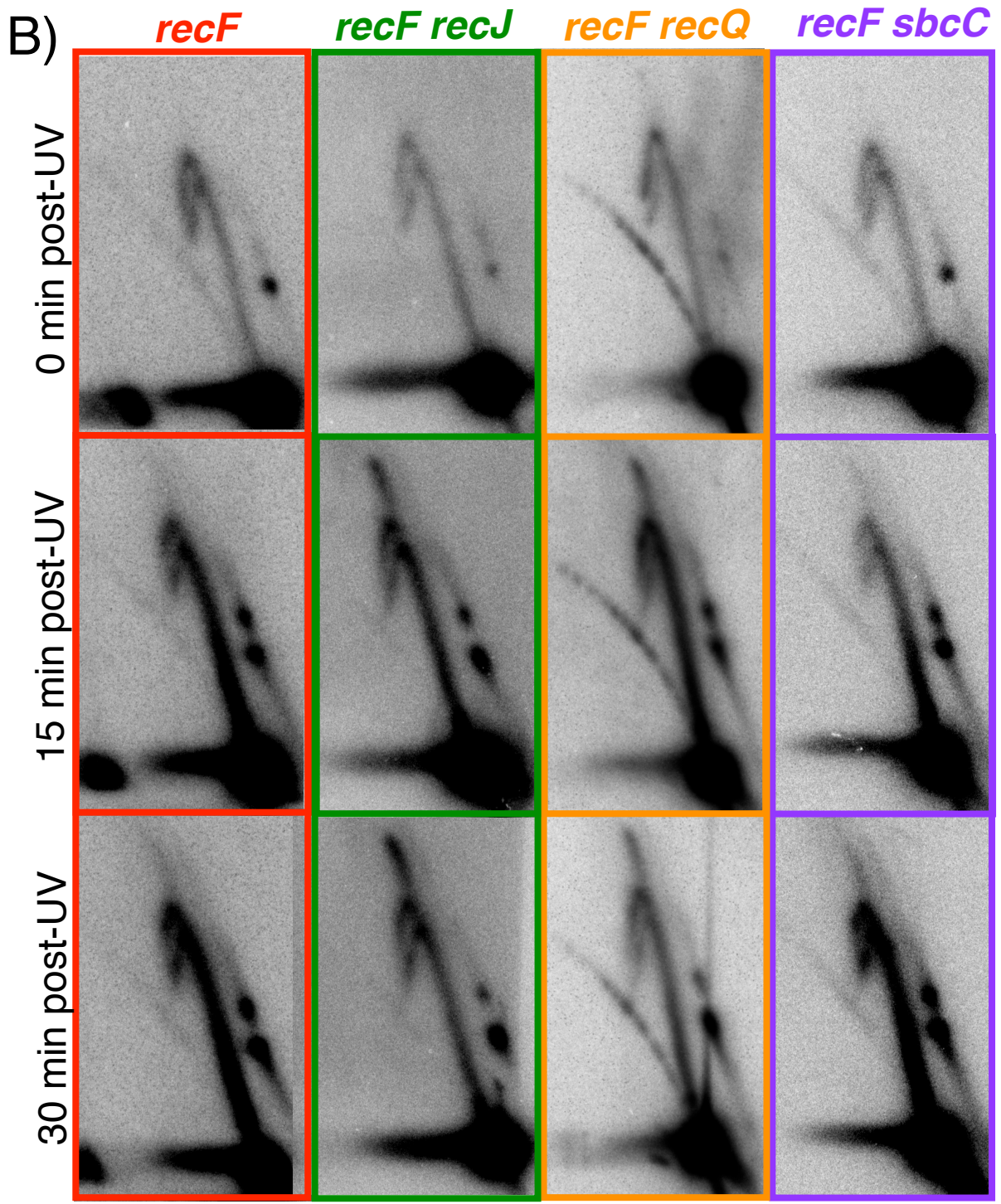
Figure 7. Current Model for Completing DNA Replication in *E. coli* and speculative human comparison. Purple, Replisome Proteins; Green, Helicase-Primase; Green Line, nascent DNA; Black Lines, Parental DNA; Red, *E. coli* Proteins; Blue, human proteins. (*E. coli* model derived in part from data presented here and in [60–62, 72, 76, 94, 96, 97]), Proposed speculative human activities derived in part from data presented in [32–37, 84, 89–92, 98]).

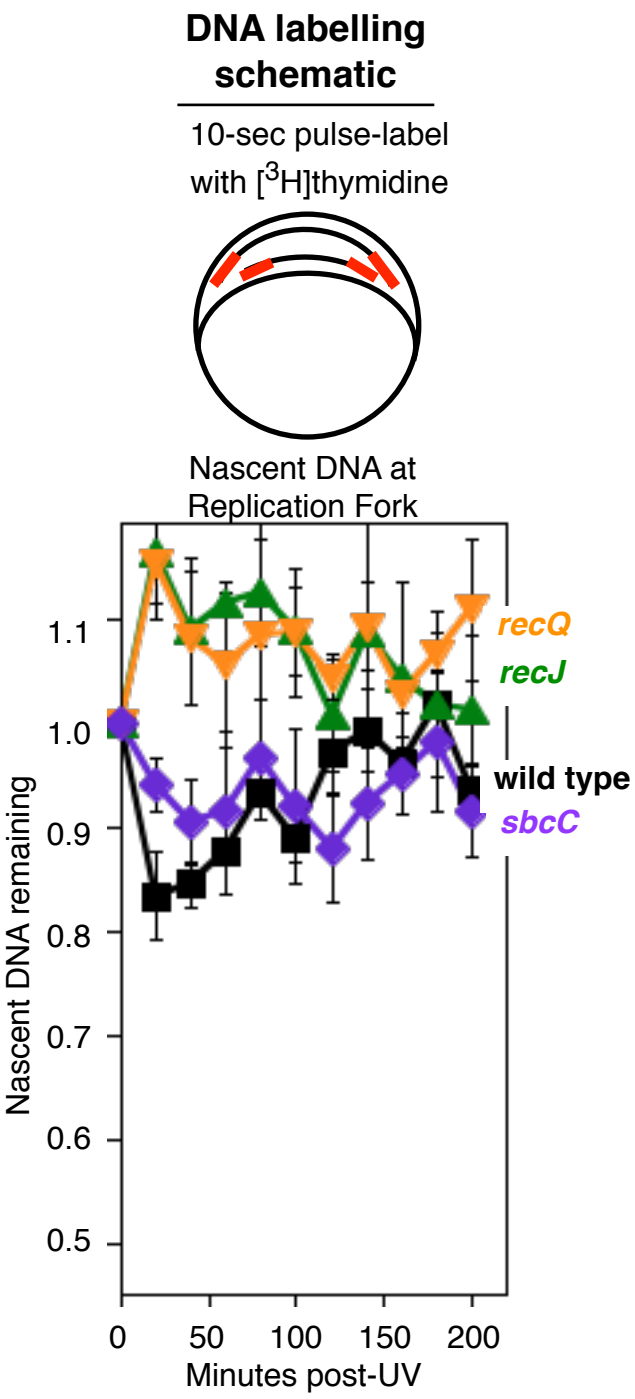
Supporting Documents:

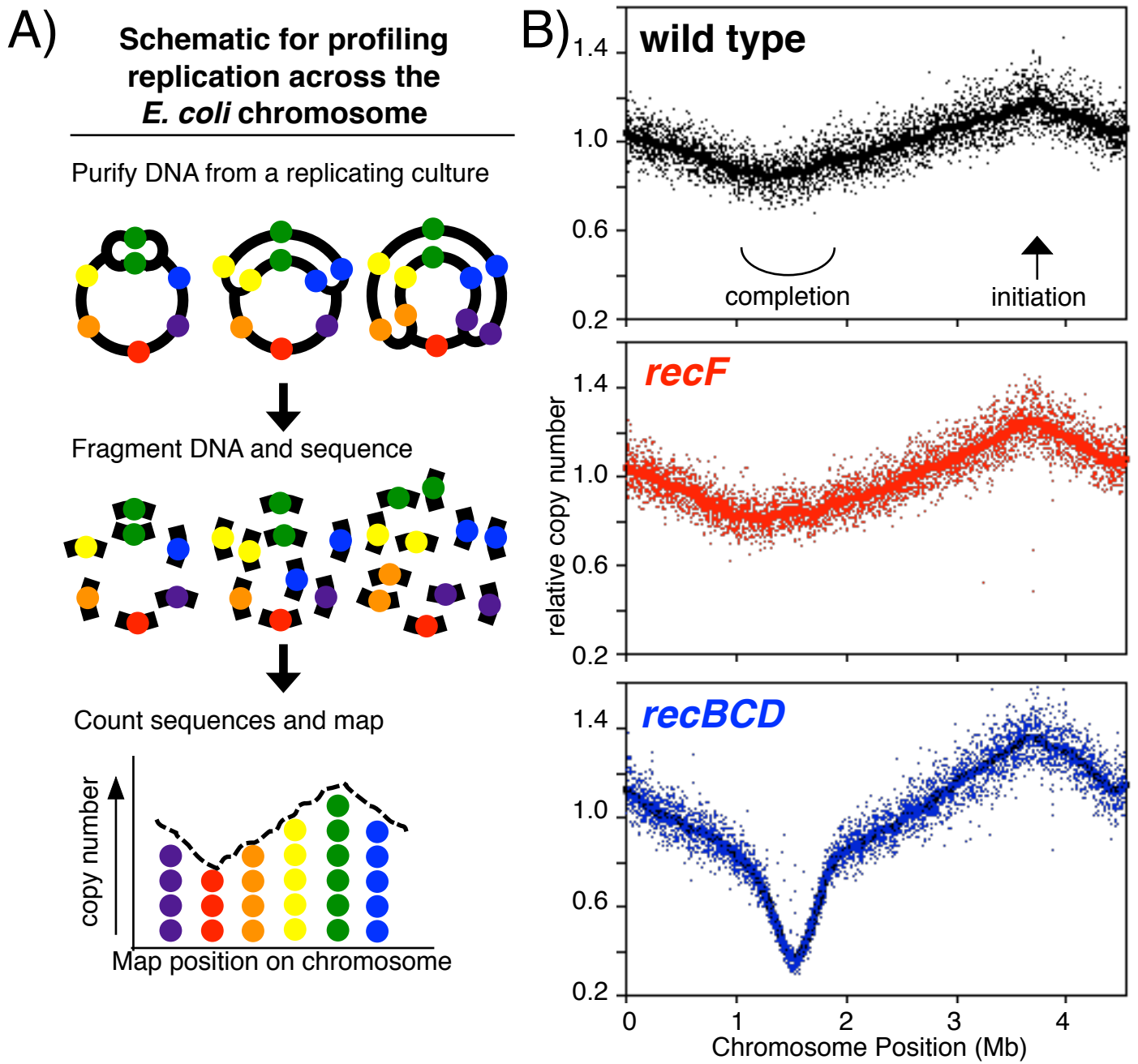
Supplementary Figure S1. Survival following UV irradiation.

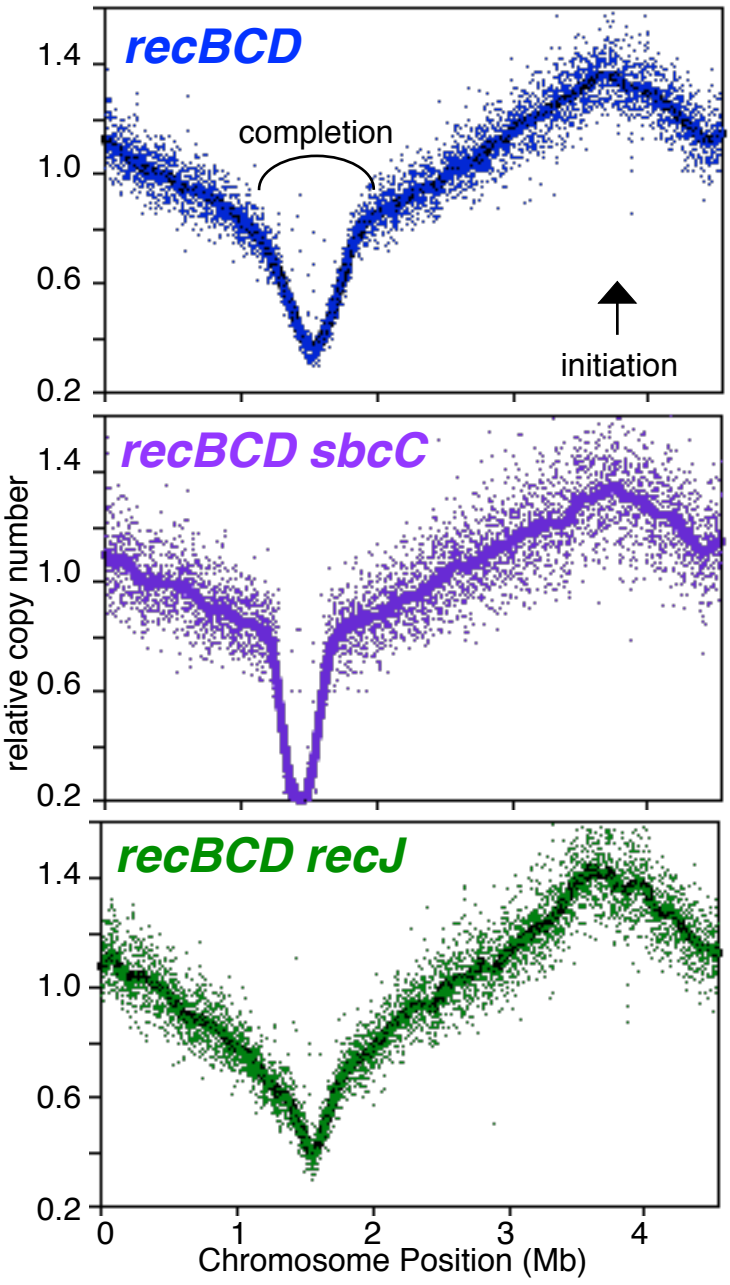

A)
DNA labelling schematic
10-sec pulse-label with [3H]thymidine
Continuous growth in [3H]thymidine
B)
Nascent DNA at Replication Fork
Total Genomic DNA
DNA remaining
1.1
1.0
0.9
0.8
0.7
0.6
0.5
wildtype
recBCD
recF
0
50
100
150
200
Minutes post-UV
C)
iii Damage-induced fork intermediates
ii Replicating fragments
i Non-replicating fragments
iii
ii
i
D)
wild type
recF
recBCD
0 min post-UV
15 min post-UV
30 min post-UV


A)
DNA labelling schematic
10-sec pulse-label with [3H]thymidine
Nascent DNA at Replication Fork
recFrecJ
recFrecQ
recF
recFsbcC
Nascent DNA remaining
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
50
100
150
200
Minutes post-UV


B)
recF
recF recJ
recF recQ
recF sbcC
0 min post-UV
15 min post-UV
30 min post-UV


DNA labelling schematic
10-sec pulse-label with [3H]thymidine
Nascent DNA at Replication Fork
recQ
recJ
wild type
sbcC
Nascent DNA remaining
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0
50
100
150
200
Minutes post-UV


A)
Schematic for profiling replication across the E. coli chromosome
Purify DNA from a replicating culture
Fragment DNA and sequence
Count sequences and map
copy number
Map position on chromosome
B)
wild type
completion
initiation
recF
recBCD
relative copy number
1.4
1.0
0.6
0.2
0
1
2
3
4
Chromosome Position (Mb)

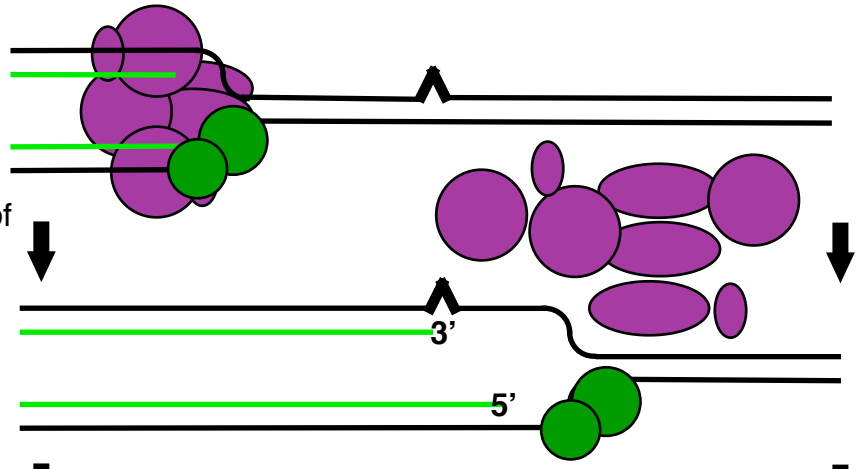

recBCD
completion
initiation
recBCD sbcC
recBCD recJ
relative copy number
1.4
1.0
0.6
0.2
0
1
2
3
4
Chromosome Position (Mb)

E. coli

Human

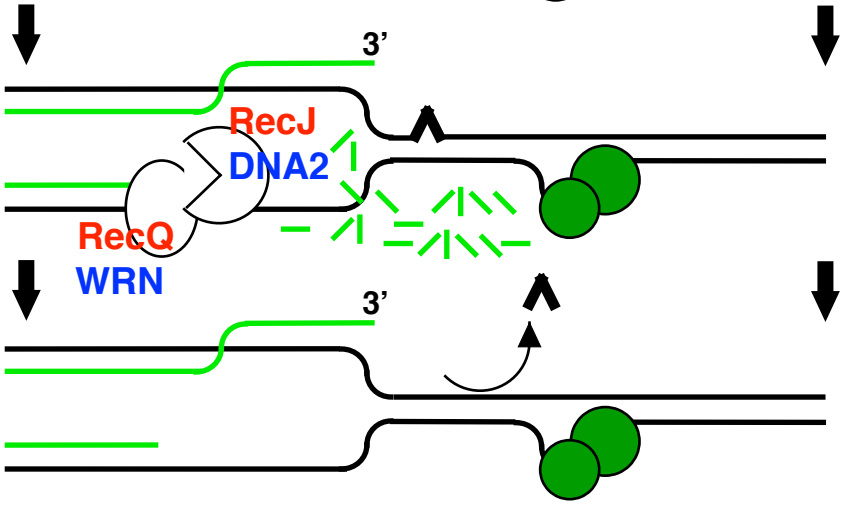
i) Replication is disrupted by UV lesions, leading to the partial disociation of replisome components from the helicase

i) Replication is disrupted by hydroxyurea, leading to the partial disociation of replisome components from the helicase



ii) The nascent lagging strand DNA is degraded by RecQ and RecJ...

ii) The nascent lagging strand DNA is degraded by WRN-DNA2...

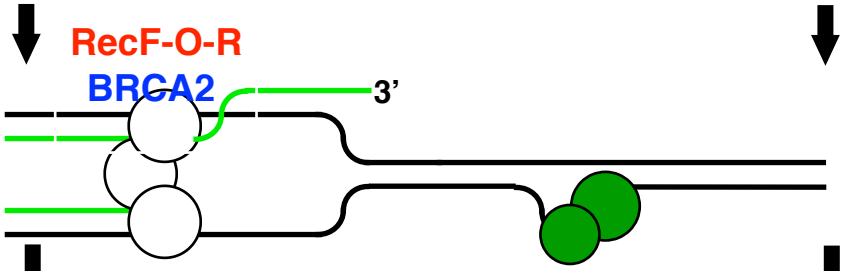


iii)... restoring the lesion containing region to a double stranded form that can be repaired.

iii)... restoring the lesion containing region to a double stranded form that could be repaired, if present.

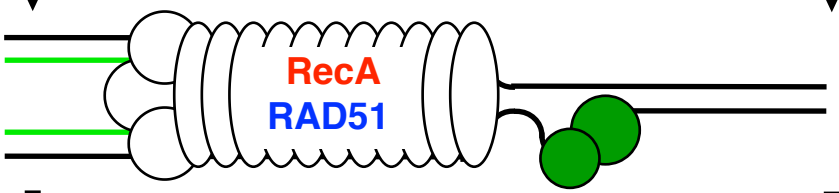
iv) RecF, -O, and -R limit the degradation....

iv) BRCA2 and other enzymes limit the degradation and promote RAD51 loading....



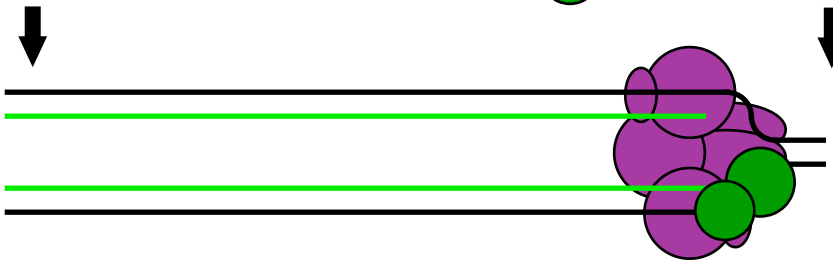
v) ...and promote RecA loading to maintain the integrity and reset the replication fork.

v) ...and promote RAD51 loading to maintain the integrity and reset the replication fork.



vi).... allowing the replisome's components to reassociate with the helicase and replication to resume.

vi).... allowing the replisome's components to reassociate with the helicase and replication to resume.

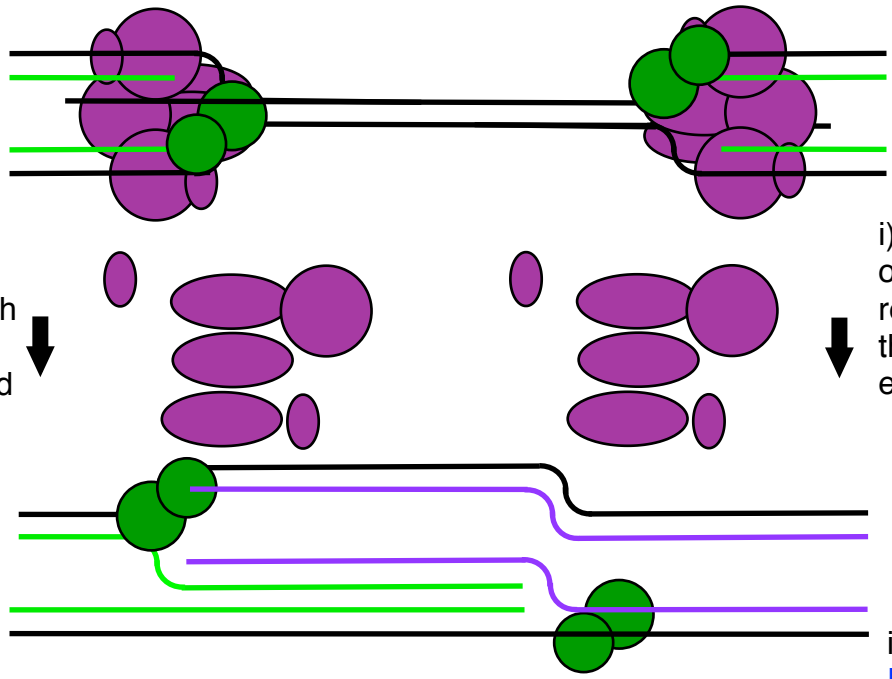


E. coli

Human

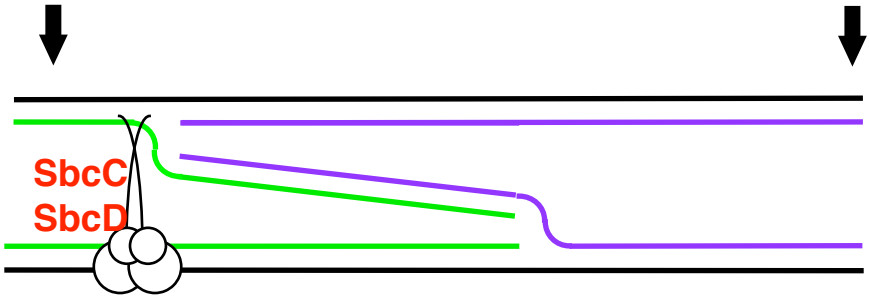
i) Replication forks converge and transiently bypass each other, leading to a partially over-replicated region on the chromsome

i) It remains unclear if over-replicated regions can arise on the chromsomes in eukaryotes



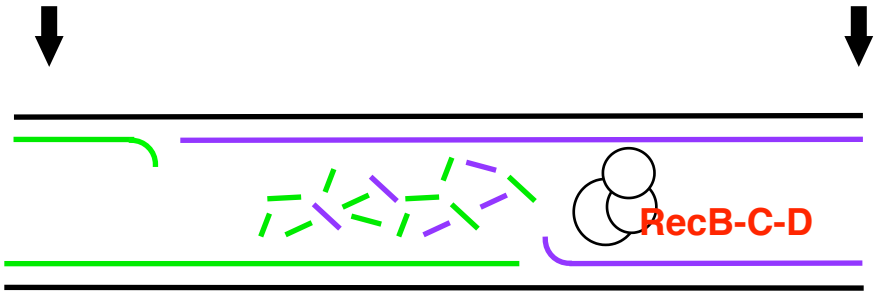
ii) SbcC and SbcD incise the hairlin-like junction, creating a double strand end...

ii) MRE11 and RAD50 share homology, but it remains unknown if they play a role in this process creating a double strand end...

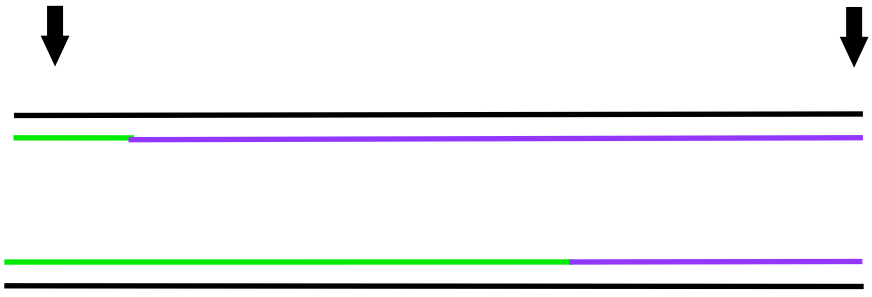


iii)... RecBCD along with Exo I, then processes and degrades the .over-replicated region.

iii) hPif1 shares homology with RecD, but there are no clear homologs of RecB-C-D. hPif1 has been previously proposed to participate in the completion reaction.



iv) before Ligase and Pol I are recruited to fill in remaining gaps and join the nascent strands....



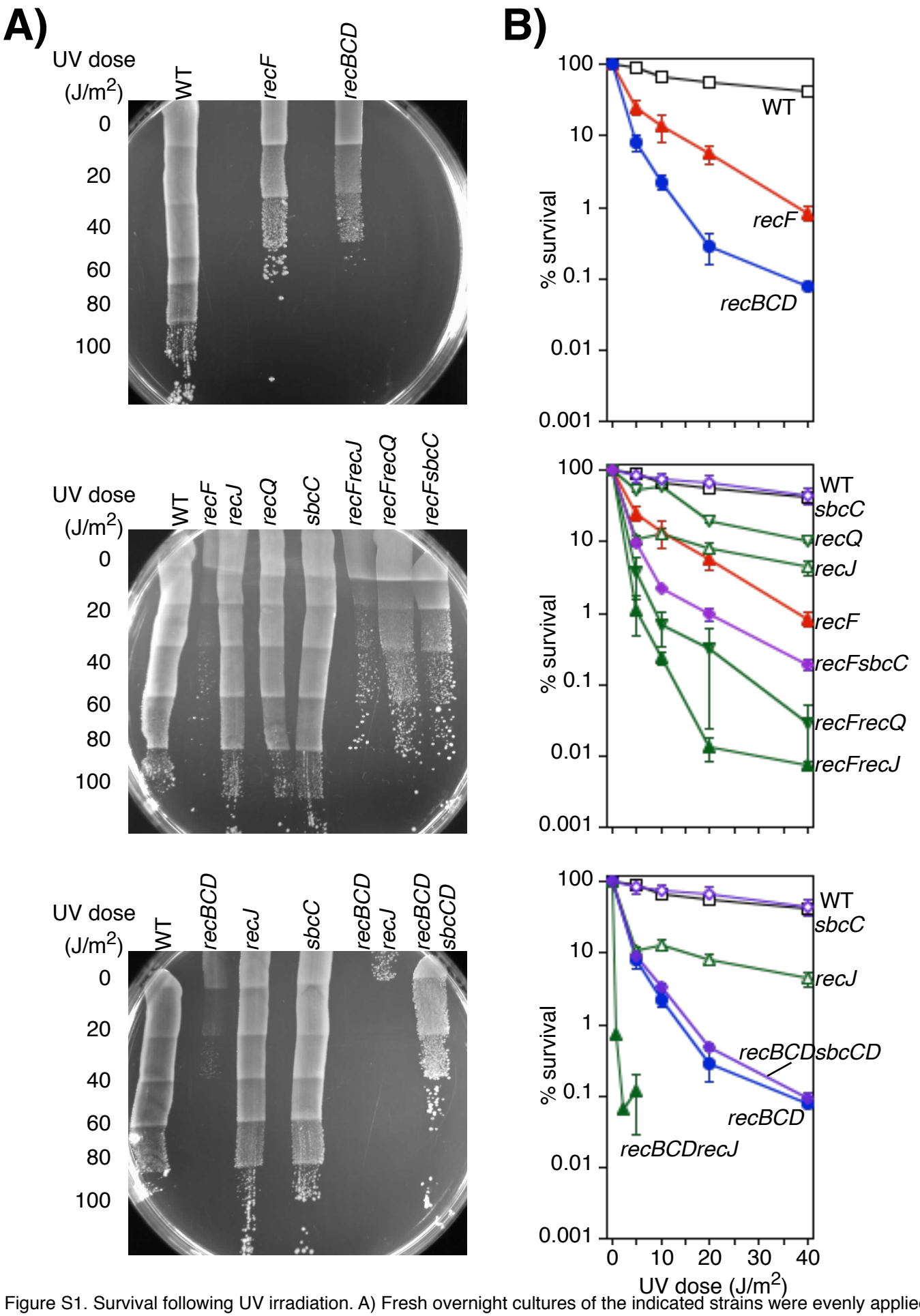
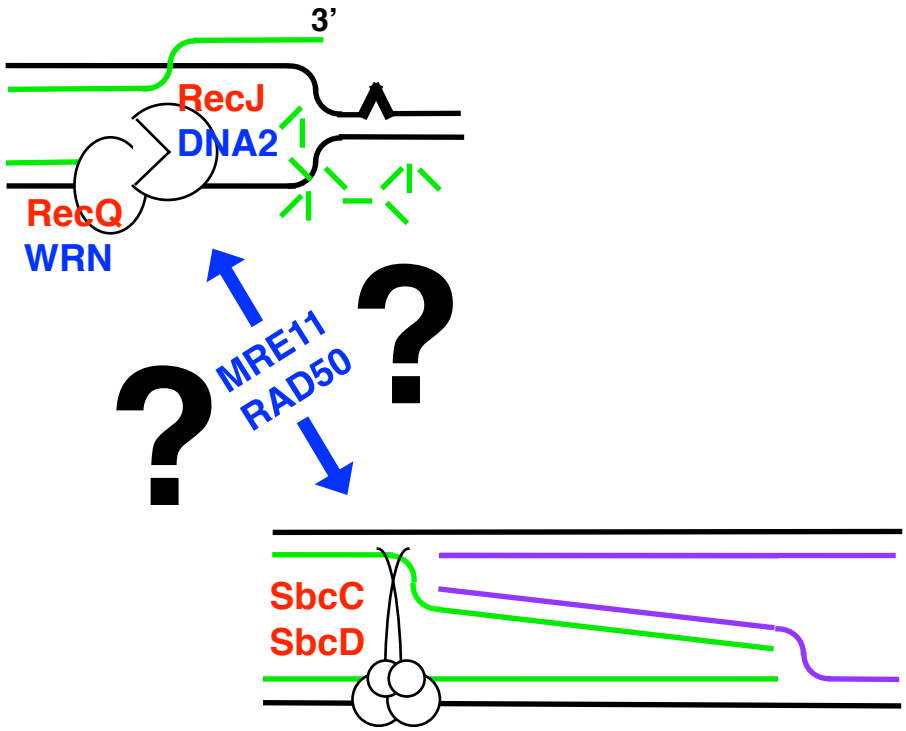


Figure S1. Survival following UV irradiation. A) Fresh overnight cultures of the indicated strains were evenly applied on a Luria-Bertani medium plate with a cotton swab. The plate was covered by a sheet of aluminum foil and placed under a 15 W germicidal lamp (254 nm; 1 J/m2/s). The foil was progressively retracted following 20 J/m2 exposures. The irradiated plate was then incubated at 37°C for overnight and photographed. B) Survival of the indicated strains following UV irradiation. Graphs represent averages of at least two independent experiments. Error bars represent the standard error of the mean.


3’
RecJ
DNA2
RecQ
WRN
MRE11
RAD50
?
?
SbcC
SbcD

BRCA2, MRE11-RAD50, and WRN-DNA2 encode human proteins that process replication forks and result in distinct genetic instabilities and cancers when mutated.

Here, we show their bacterial homologs act on unique replication forks substrates- those at DNA damage sites or as replication completes, and discuss their possible functional conservation in humans.