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Fork regression is an active helicase-driven pathway in bacteriophage T4

David T. Long[†] *& Kenneth N. Kreuzer*[+]

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA

Reactivation of stalled replication forks requires specialized mechanisms that can recognize the fork structure and promote downstream processing events. Fork regression has been implicated in several models of fork reactivation as a crucial processing step that supports repair. However, it has also been suggested that regressed forks represent pathological structures rather than physiological intermediates of repair. To investigate the biological role of fork regression in bacteriophage T4, we tested several mechanistic models of regression: strand exchange-mediated extrusion, topology-driven fork reversal and helicase-mediated extrusion. Here, we report that UvsW, a T4 branch-specific helicase, is necessary for the accumulation of regressed forks *in vivo*, and that UvsW-catalysed regression is the dominant mechanism of origin-fork processing that contributes to double-strand end formation. We also show that UvsW resolves purified fork intermediates *in vitro* by fork regression. Regression is therefore part of an active, UvsW-driven pathway of fork processing in bacteriophage T4.

Keywords: DNA damage; fork regression; helicase; replication

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INTRODUCTION

It has become evident that replication forks routinely stall and become inactivated (Mirkin & Mirkin, 2007). Inactivation probably involves the dissociation of replisome components, which presumably facilitates DNA repair pathways. However, exposed fork structures are inherently fragile, contributing to the formation of DNA breaks, genome instability and cell death. Reactivation of stalled forks requires specialized processing mechanisms that can both recognize the fork structure and facilitate reloading of the replisome.

Several reports argue that replication fork regression can be involved in fork processing pathways *in vivo* (Michel *et al*, 2001; Courcelle *et al*, 2003; Long & Kreuzer, 2008). Fork regression involves extrusion and annealing of leading and lagging strands from the template DNA, resulting in a double-stranded DNA end (DSE) and a Holliday junction. Some models of fork reactivation invoke fork regression as a crucial processing step, including the strand switching-mediated lesion bypass (Higgins *et al*, 1976), replisome/branch clearance for lesion repair (McGlynn & Lloyd, 2002) and recombination-dependent replication (Michel *et al*, 2001).

Other studies have suggested that regressed forks represent pathological structures, rather than physiological intermediates of repair (Lopes *et al*, 2001; Sogo *et al*, 2002; Meister *et al*, 2005). The accumulation of aberrant DNA structures—including regressed forks—is correlated with cell death in *rad53* mutant (checkpoint-defective) budding yeast cells, which led to the suggestion that stalled forks are normally stabilized during the checkpoint response to prevent spontaneous regression (Lopes *et al*, 2001; Sogo *et al*, 2002; Yoon *et al*, 2004). However, an alternative interpretation is that stalled forks are actively regressed to facilitate reactivation, but that regressed forks are extremely transient intermediates in wild-type cells. In this view, the accumulation of regressed intermediates in the *rad53* mutant is due to a defect in regressed fork processing by some Rad53-dependent pathway.

Understanding the mechanism of fork regression should shed light on its biological role—that is, pathological structures versus physiological intermediates. Although the requirements for *in vivo* fork regression are still unknown, several models of regression have emerged, mostly from *in vitro* experiments.

(i) Strand exchange-mediated extrusion. Strand exchange proteins might localize to regions of single-stranded DNA within stalled fork structures and promote strand exchange between the two arms of a fork intermediate, leading to extrusion and annealing of the two daughter strands. This reaction has been shown *in vitro* on model fork structures with *Escherichia coli* RecA (Robu *et al*, 2001), human Rad51 (Yoon *et al*, 2004) and bacteriophage T4 UvsX (Kadyrov & Drake, 2004). Strand exchange between fork arms has also been proposed to function in fork stability by preventing regression. In this case, a limited region of strand exchange is proposed to link the fork arms together and prevent spontaneous fork regression or inappropriate recombination reactions (Donaldson *et al*, 2006).

(ii) Topology-driven fork reversal. During replication fork progression, positive supercoils can accumulate ahead of the fork if

Department of Biochemistry, Duke University Medical Center, Box 3711, Durham, North Carolina 27710, USA

[†]Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, C-2 226 Boston, Massachusetts 02115, USA

[+]Corresponding author. Tel: +1 919 684 6466; Fax: +1 919 684 6525; E-mail: kenneth.kreuzer@duke.edu

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topoisomerase activity is insufficient (for review, see Schvartzman & Stasiak, 2004). This positive supercoiling can be relieved by spontaneous fork regression, as shown *in vitro* with partly replicated plasmid intermediates (Postow *et al*, 2001).
(iii) Helicase-mediated extrusion. Several DNA helicases have been shown to promote regression of model forks *in vitro* (McGlynn *et al*, 2001; Machwe *et al*, 2006; Ralf *et al*, 2006; Blastyák *et al*, 2007; Li *et al*, 2008). In addition, Michel *et al* (2001) have implicated the *E. coli* helicase RecG in a RuvAB-mediated fork regression pathway *in vivo* based on genetic results and an indirect assay involving RuvC-dependent chromosomal breakage. Similar approaches argue that RuvAB participates in fork regression, either through regressed fork stabilization (Seigneur *et al*, 1998) or by directly catalysing fork regression (Masson *et al*, 2008).

To analyse pathways of fork processing in bacteriophage T4, we have used the *ori(34)* origin-fork as a model fork intermediate (Long & Kreuzer, 2008). The origin-fork is formed naturally during T4 infection when one fork exits the origin region but the retrograde fork has not yet started. The amount of origin-fork detected as a function of time presumably depends on various factors such as the efficiency of origin R-loop formation and removal, the efficiency of leading-strand priming from the R-loop and the efficiency of retrograde fork initiation. Retrograde fork initiation is similar to stalled fork reactivation, and thus the origin-fork allows simplified analysis of fork processing mechanisms without the complications of replication inhibitors or DNA damage. Previously, we have shown that the origin-fork undergoes regression *in vivo*, and that regression supports two mechanisms of fork processing (Long & Kreuzer, 2008).

Here, we show that the T4-encoded UvsW protein is necessary for the accumulation of regressed origin-fork intermediates *in vivo* and catalyses fork regression *in vitro*. UvsW is a T4 branch-specific helicase that shares structural homology to the eukaryotic SF2 helicase, Rad54 (Kerr *et al*, 2007). We also show that UvsW-driven fork regression is the dominant mechanism of DSE formation at the origin-fork. These results argue that regression is part of a biologically relevant pathway of fork processing in bacteriophage T4.

RESULTS

uvsW is required for accumulation of regressed forks

Previously, we have detected regressed origin-forks at early times of T4 infection, and observed that the amount of regressed forks increased in a gene *46* mutant infection (Long & Kreuzer, 2008). Gene *46* encodes an essential component of the gp46/47 exonuclease–ATPase complex, which is a member of the Rad50/Mre11 family. gp46/47 is known to degrade DSEs *in vivo* (see Kreuzer, 2000), explaining the increase in regressed origin-fork accumulation. Therefore, we used a *46*$^-$ background to test particular gene mutations for their effect on regressed origin-fork accumulation.

To test each of the three models of fork regression (see above), we introduced *uvsX*, gene *39*, *dda* and *uvsW* mutations into the T4 *46*$^-$ background. *uvsX* encodes the T4 strand-exchange protein, a member of the RecA/Rad51/RadA family of recombinases. Gene *39* encodes a required subunit of the T4 type II topoisomerase. According to the topology model, mutation of gene *39* might be expected to increase fork regression owing to the accumulation of unresolved positive DNA supercoils. T4 encodes three DNA helicases: gp41, Dda and UvsW. gp41 is the replicative helicase and is required for extensive, coordinated replication of both the leading and lagging strands. Therefore, we could not investigate whether regressed origin-forks accumulate in the absence of gp41. Dda is a well-characterized DNA helicase, the *in vivo* function of which is unclear. UvsW, a T4 helicase that promotes branch migration of Holliday junctions (Webb *et al*, 2007), is perhaps the best candidate for an enzyme that catalyses fork regression. This protein was shown to resolve Y-shaped DNA intermediates *in vitro*, but the intermediates and products were not identified and thus the mechanism of resolution was ambiguous.

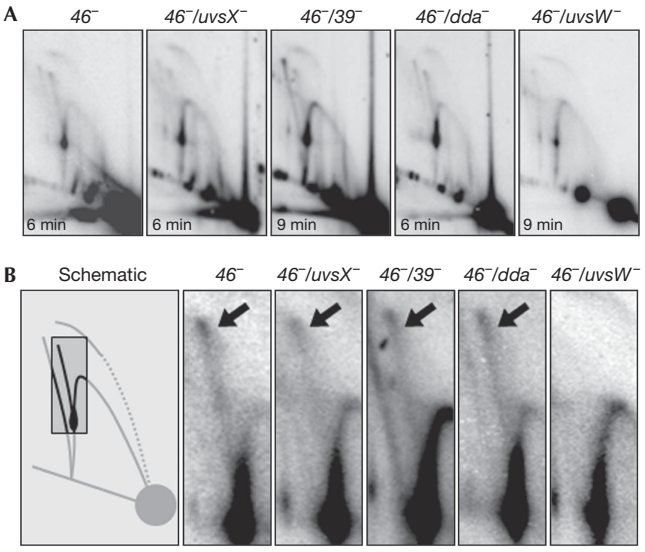

Fig 1 | Requirements for origin-fork regression *in vivo*. (**A**) Total DNA from the indicated T4 infections was digested with *Pac*I and separated by two-dimensional gel electrophoresis. The time point shown for each infection was the time of maximal origin-fork accumulation (see Long & Kreuzer, 2008; complete time courses in supplementary Fig 1 online). Gels were visualized by Southern blot with a probe specific to the *ori(34)* region. (**B**) The two-dimensional gel region containing the regressed origin-fork arc is shown at increased magnification and contrast, with the regressed origin-fork arc indicated by an arrow.

DNA intermediates from each mutant infection were assayed for regressed fork accumulation using two-dimensional agarose gel electrophoresis (Fig 1). In two-dimensional gels, regressed origin-fork intermediates form an arc that is similar in appearance to the X-arc but that originates from the origin-fork position on the Y-arc (Fierro-Fernandez *et al*, 2007; Long & Kreuzer, 2008).

Mutation of genes *uvsX*, *39* or *dda* had no apparent effect on regressed origin-fork accumulation (Fig 1). Although these results indicate that UvsX and Dda are not required for fork regression *in vivo*, they do not rule out the possibility that topology has a function in the regression of authentic stalled replication forks (as opposed to the origin-forks studied here). The most notable result is that the *46*$^-$/*uvsW*$^-$ infections showed no discernible accumulation of regressed origin-fork intermediates, showing directly that UvsW is required for regression *in vivo* (Fig 1).

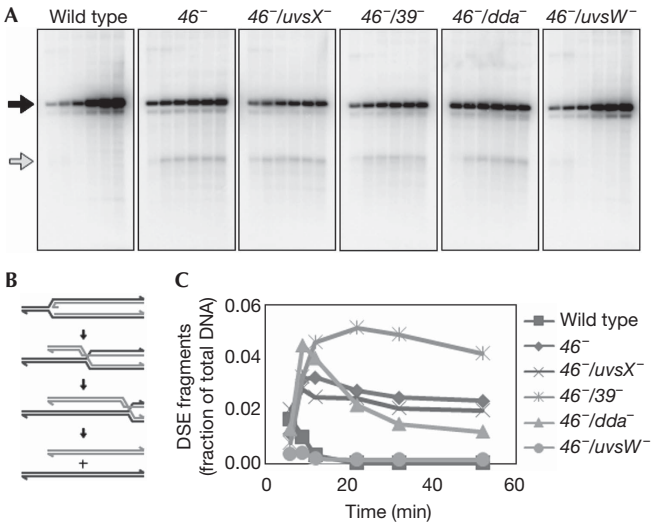

Fig 2 | Accumulation of double-stranded DNA end fragments in the *ori(34)* region. (**A**) Total DNA from the indicated T4 infections at 6, 9, 12, 22, 32 and 52 min post-infection was digested with *Ase*I and separated by agarose gel electrophoresis. DNA was visualized by Southern blot with a probe specific to the region downstream from *ori(34)*. Full-length restriction fragments and the 1.48-kb DSE fragment are indicated by black and gray arrows, respectively. (**B**) Schematic diagram depicting the formation of DSE fragments following origin-fork regression and extensive HJ branch migration. (**C**) Relative DSE fragment accumulation over time for each infection as a fraction of total DNA (average of three experiments). DSE, double-stranded DNA end; HJ, Holliday junction.

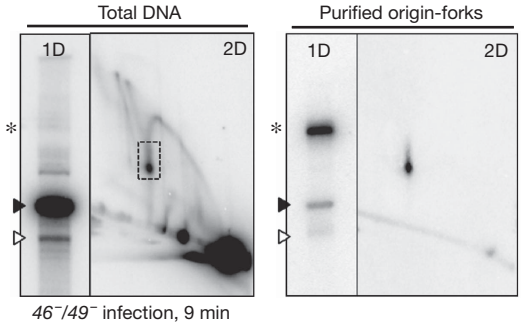

Fig 3 | Origin-fork purification. Total DNA from a *46⁻/49⁻* phage infection (9 min post-infection; left) and purified origin-fork intermediates (right) were analysed by one- and two-dimensional gel electrophoresis followed by Southern blotting with an *ori(34)* probe. The one-dimensional gel position of the origin-fork is indicated by an asterisk, and the 6.2- and 5.2-kb linear fragments are indicated by closed and open arrowheads, respectively. 1D, one dimensional; 2D, two dimensional.

Previously, we found that in a *46⁻/49⁻* background, regressed origin-forks accumulate to a level about twice that found in *46⁻* infections (Long & Kreuzer, 2008). This result implied that the product of gene *49*, EndoVII, cleaves Holliday junctions formed at some of the regressed origin-forks. The triple mutant *46⁻/49⁻/uvsW⁻* showed no discernible accumulation of regressed origin-forks (supplementary Fig 1 online), supporting the conclusion that UvsW is required for regression *in vivo*.

uvsW is required for accumulation of *ori(34)* fragments

Fork stalling is often associated with the appearance of DSEs, presumably owing to fork processing by any of several possible mechanisms: fork breakage, regression and head-to-tail fork collisions. Previously, we have shown that origin-fork accumulation is associated with the formation of DSEs, with a 1.48-kb DSE fragment resulting from restriction enzyme digestion with *Ase*I (Long & Kreuzer, 2008). This DSE fragment could be generated by origin-fork regression if the Holliday junction created by regression undergoes branch migration past the *Ase*I restriction site (Fig 2B).

To test the requirements for DSE formation, we analysed the accumulation of *ori(34)* DSE fragments in *46⁻* infections—as noted above, inactivation of gene *46* prevents degradation of DSEs (Fig 2). DSE fragments accumulated in the *46⁻/uvsX⁻*, *46⁻/39⁻* and *46⁻/dda⁻* infections to levels similar to that of a *46⁻* single mutant infection. However, *46⁻/uvsW⁻* infections were almost completely deficient for DSE fragment accumulation, implying that UvsW-catalysed regression is the dominant mechanism of origin-fork processing that leads to DSE formation.

UvsW protein resolves origin-forks by regression *in vitro*

Previously, we reported that UvsW is able to resolve Holliday junction-containing substrates *in vitro* by promoting extensive branch migration (Webb *et al*, 2007). In that study, we also found that UvsW resolves Y-shaped intermediates *in vitro*. However, as the substrate was total DNA from a T4 infection—containing various T4-modified DNA intermediates—we could not readily identify the products or intermediates of resolution.

To analyse the mechanism of fork resolution by UvsW, we introduced inter-strand DNA crosslinks into total DNA before treatment with UvsW protein and visualization by a two-dimensional gel (supplementary Fig 2 online). Increasing amounts of crosslinks prevented the complete resolution of both X- and Y-shaped intermediates. In addition, a collection of partly resolved intermediates accumulated within a cone-shaped region between the X- and Y-arcs. This accumulation is consistent with the expected migration pattern for partly regressed fork structures of various sizes (see schematic diagram, supplementary Fig 2 online; Fierro-Fernandez *et al*, 2007).

To perform a simpler analysis on the mechanism of fork resolution by UvsW, we purified the origin-fork intermediate so that it became the main DNA form detected by Southern blotting with an *ori(34)* probe. This was accomplished by separating total DNA samples from a T4 infection by using a variation of the two-dimensional gel electrophoresis procedure and extracting DNA from gel slices. Total DNA and the purified origin-fork intermediates are compared in Fig 3, and visualized by both one- and two-dimensional gels (with Southern blotting). In addition to the intact origin-fork intermediate, the preparation also contained small amounts of 6.2- and 5.2-kb linear fragments, which correspond to the full-length linear molecule and one arm of the origin-fork, respectively. These fragments presumably arise from origin-fork breakage during preparation and/or co-purification from total DNA.

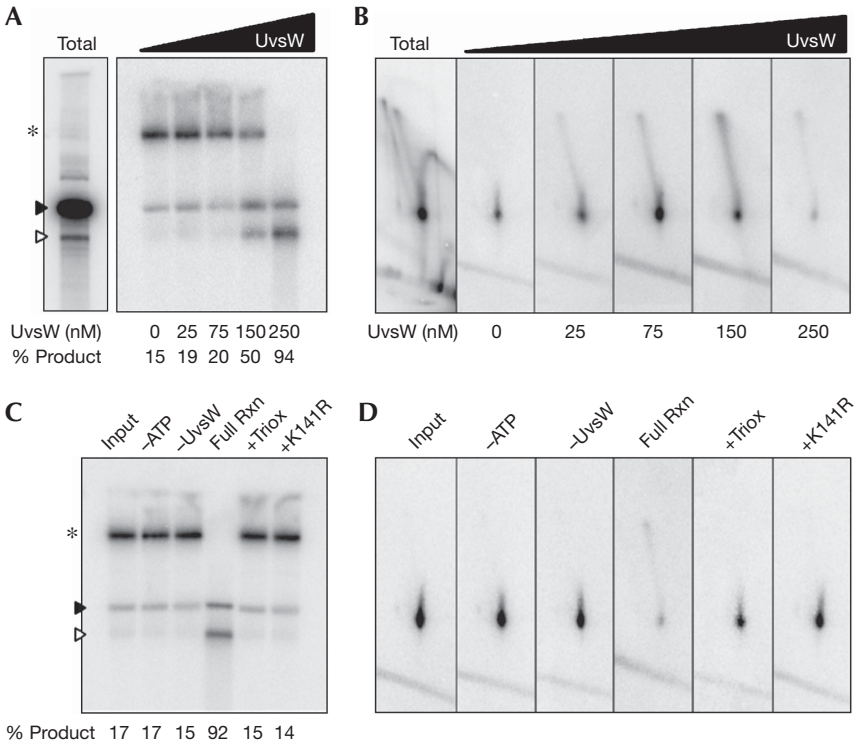

Fig 4 | UvsW resolves purified origin-forks by fork regression. (**A,B**) Purified origin-fork intermediates were treated with increasing concentrations of UvsW protein for 30 min at 37 °C in 1 × reaction buffer. Identical reactions were analysed by (**A**) one-dimensional and (**B**) two-dimensional gels. (**C,D**) Purified origin-fork intermediates were treated with UvsW (Input, −ATP, Full Rxn and +Triox) or UvsW-K141R (+K141R) proteins (250 nM) for 30 min at 37 °C in 1 × reaction buffer in the presence of ATP and in the absence of interstrand DNA crosslinks unless otherwise indicated (−ATP and +Triox, respectively). Identical reactions were analysed by (**C**) one-dimensional and (**D**) two-dimensional gels. All gels were visualized by Southern blot with an *ori(34)* probe. The one-dimensional gel position of the origin-fork is indicated by an asterisk, and the 6.2- and 5.2-kb linear fragments are indicated by closed and open arrowheads, respectively. Rxn, reaction; Triox, trioxsalen.

By using the purified origin-fork intermediates, we analysed the mechanism of fork resolution by UvsW *in vitro*. Increasing amounts of UvsW resolved the origin-fork into 6.2- and 5.2-kb linear fragments as visualized by a one-dimensional gel (Fig 4A). When identical reactions were analysed by using two-dimensional gels, increasing amounts of UvsW were found to generate a regressed fork arc that was identical to that seen in Fig 1 (Fig 4B; arc from total DNA is also shown for comparison). At the highest concentration of UvsW (250 nM), most origin-fork intermediates were resolved to linear fragments not visible in the two-dimensional gel image. As determined using both one- and two-dimensional gels, resolution of origin-forks by UvsW required the presence of ATP and was blocked by inter-strand DNA crosslinks in the substrate (Fig 4C,D). In addition, purified UvsW protein with a mutation in the Walker A motif (K141R; deficient in ATP hydrolysis and branch migration) was unable to carry out origin-fork resolution (Fig 4C,D). These results show that UvsW promotes resolution of fork intermediates through fork regression, followed by extensive branch migration until the resulting Holliday junction falls off the end of the restriction fragment.

DISCUSSION

Here, we have shown that the branch-specific helicase UvsW is necessary for the accumulation of regressed origin-forks *in vivo*, and that the UvsW protein is sufficient for origin-fork regression *in vitro*. In addition, *46*$^{-}$*/uvsW*$^{-}$ infections are almost completely deficient for DSE fragment accumulation. Thus, fork regression is the dominant mechanism of fork processing that leads to DSE formation, rather than fork breakage or head-to-tail fork collisions. Previously, we reported that regression supports two mechanisms of processing that can lead to fork reactivation—regressed DSE degradation by gp46/47 and Holliday-junction cleavage by T4 EndoVII (Long & Kreuzer, 2008). Taken together, these results indicate that UvsW-catalysed regression is part of an active helicase-driven pathway of fork processing that can stimulate fork reactivation in T4. This conclusion is supported by the fact that UvsW-deficient mutants—including deletion and K141R substitution—are hypersensitive to hydroxyurea, which causes fork stalling owing to nucleotide starvation (Carles-Kinch *et al*, 1997, and references therein). The hypersensitivity indicates that UvsW has a crucial function in reactivating stalled forks after hydroxyurea treatment, although further experiments are needed to test directly whether this crucial function involves UvsW-promoted fork regression.

Origin-fork resolution is severely delayed in *46*$^{-}$*/49*$^{-}$ infections that are deficient in regressed DSE degradation by gp46/47 and Holliday-junction cleavage by EndoVII (Long & Kreuzer, 2008). Interestingly, the *uvsW* mutation seems to rescue this delay

(supplementary Fig 1 online), suggesting that alternative processing mechanisms are sufficient for wild-type levels of origin-fork resolution if fork regression by UvsW is prevented. This alternative processing mechanism could involve direct loading of the replicative helicase gp41 by the helicase loading protein gp59. These observations might relate to the association of genome instability and cell death with replication fork regression (Lopes *et al*, 2001). Once a fork structure becomes regressed, it might be 'committed' to resolution by specific processing mechanisms and the absence of such mechanisms might be particularly detrimental.

In addition to UvsW, several other helicases have recently been shown to promote fork regression *in vitro* (McGlynn *et al*, 2001; Machwe *et al*, 2006; Ralf *et al*, 2006; Blastyák *et al*, 2007; Li *et al*, 2008). Of particular interest are WRN and BLM, which are implicated in human diseases characterized by premature ageing and cancer predisposition, respectively. Although the biological role of these proteins has not been established *in vivo*, the implication is that fork regression might have a crucial function in genome stability by facilitating stalled fork processing and reactivation. Indeed, a crucial function of fork regression *in vivo* might be to avoid the formation of overt DNA breaks that contribute to genome instability.

METHODS

Bacterial phage and strains. The bacterial host for phage infections is a derivative of *E. coli* CAG12135 with the following additional mutations: *acrA*::Tn*10-kan*, *recA*::Tn*10-cam* and *recD*. The phage T4 strains used in this study are derivatives of strain K10 (*am*B262 (gene *38*), *am*S29 (gene *51*), *nd*28 (*denA*), *rII*PT8 (*rII-denB* deletion); Selick *et al*, 1988). The additional phage mutations used here include: *am*B14 (gene *46*), *am*E727 (gene *49*), *am*HL628 (gene *59*), *dda*Δ (T4 coordinates 10 449–10 629), *am*11 (*uvsX*), *am*N116 (gene *39*) and *uvsW*^{am} (CT→AG mutation at T4 coordinates 113 075–113 076, constructed using the T4 I/S system (Selick *et al*, 1988)].

T4 infection and DNA analyses. T4 infections, DNA purifications and gel analysis of DNA were carried out essentially as described by Long & Kreuzer (2008). Details are presented in the supplementary information online.

Purification of origin-fork intermediates. Total DNA from a 20 ml infection (*46*$^{-}$/*49*$^{-}$ phage, 9 min post-infection) was prepared as described in the supplementary information online and treated with *Pac*I. For the one-dimensional gel, digested DNA was loaded into a wide, trough-shaped well and subjected to electrophoresis at 0.75 V/cm for 72 h at 21 °C. This extended one-dimensional gel produces greater separation of DNA intermediates and allows selection of two-dimensional gel slices that closely border the origin-fork position (based on the migration of size markers). The two-dimensional gel was then cast and run normally, and the gel was sliced to produce a narrow region that contains the origin-forks. DNA was then extracted from the gel slices by electro-elution into dialysis tubing, followed by ethanol precipitation and resuspension in TE buffer.

UvsW reactions. UvsW protein was purified as described previously (Webb *et al*, 2007). Enzyme reactions were carried out in 1 × reaction buffer (50 mM NaCl, 20 mM Tris–HCl (pH 7.8), 1 mM DTT, 0.1 mg/ml BSA, 3.5 mM $MgCl_2$ and 10 mM ATP) at 37 °C for 30 min with the indicated concentration of UvsW. DNA was then extracted sequentially with phenol and chloroform–isoamyl alcohol (24:1) to remove any DNA-associated proteins. Crosslinked samples were prepared by incubating DNA with trioxsalen (200 nM; Sigma-Aldrich, St Louis, MO, USA) for 1 h, followed by exposure to long-wave ultra-violet light for 20 min. Crosslinked samples were then dialysed against TE buffer for 1 h at 4 °C.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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