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The Werner and Bloom Syndrome Proteins Help Resolve Replication Blockage by Converting (Regressed) Holliday Junctions to Functional Replication Forks

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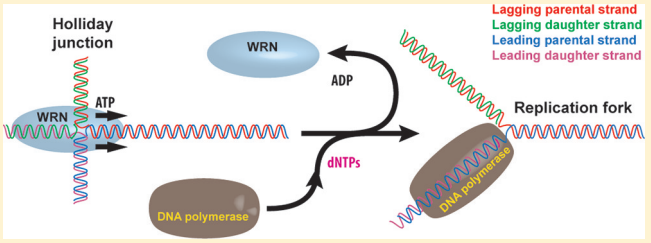
Supporting Information

ABSTRACT: Cells cope with blockage of replication fork progression in a manner that allows DNA synthesis to be completed and genomic instability minimized. Models for resolution of blocked replication involve fork regression to form Holliday junction structures. The human RecQ helicases WRN and BLM (deficient in Werner and Bloom syndromes, respectively) are critical for maintaining genomic stability and thought to function in accurate resolution of replication blockage. Consistent with this notion, WRN and BLM localize to sites of blocked replication after certain DNA-damaging treatments and exhibit enhanced activity on replication and recombination intermediates. Here we examine the actions of WRN and BLM on a special Holliday junction substrate reflective of a regressed replication fork. Our results demonstrate that, in reactions requiring ATP hydrolysis, both WRN and BLM convert this Holliday junction substrate primarily to a four-stranded replication fork structure, suggesting they target the Holliday junction to initiate branch migration. In agreement, the Holliday junction binding protein RuvA inhibits the WRN- and BLM-mediated conversion reactions. Importantly, this conversion product is suitable for replication with its leading daughter strand readily extended by DNA polymerases. Furthermore, binding to and conversion of this Holliday junction are optimal at low $MgCl_2$ concentrations, suggesting that WRN and BLM preferentially act on the square planar (open) conformation of Holliday junctions. Our findings suggest that, subsequent to fork regression events, WRN and/or BLM could re-establish functional replication forks to help overcome fork blockage. Such a function is highly consistent with phenotypes associated with WRN- and BLM-deficient cells.



RecQ helicases are critical for maintaining genomic stability, although their precise molecular functions are still unclear. There are five human RecQ family members (RECQ1, BLM, WRN, RECQ4, and RECQ5), and loss of function of BLM, WRN, and RECQ4 proteins is associated with Bloom (BS), Werner (WS), and Rothmund-Thomson (RTS) syndromes, respectively.[1−3] A hallmark feature of all these syndromes is an increased incidence of cancer that is particularly prominent in BS. In addition to an elevated predisposition particularly to soft tissue sarcomas and osteosarcomas, WS is also characterized by early onset and an increased frequency of many other age-related phenotypes.[4,5] A high incidence of osteosarcoma and abnormalities in skeletal development and skin pigmentation are associated with RTS.[2,6] At the cellular level, loss of BLM, WRN, or RECQ4 function results in an increased number of chromosomal aberrations that likely underlie the elevated cancer incidence of these syndromes. However, BS, WS, and RTS phenotypes differ significantly, suggesting these enzymes have some nonredundant functions.

RecQ members share extensive homology within conserved sequence motifs characteristic of DNA-dependent ATPases and helicases, and most possess 3′ to 5′ nucleic acid unwinding activity. WRN and BLM have very similar DNA substrate specificities, preferring to act on complex DNA structures, including forks, bubbles, D-loops, triplexes, Holliday junctions, and G-quartets.[7] Although not as well-characterized, RECQ4 has weak helicase activity that is stimulated by including complementary DNA strands in unwinding reactions.[8] Surprisingly, WRN, BLM, RECQ4, and several other RecQ helicases facilitate the annealing of complementary DNA strands.[9−12] Under certain circumstances, RecQ helicases (including WRN and BLM) can coordinate their unwinding and annealing activities to perform strand exchange.[13,14] These biochemical studies suggest that at least a subset of human RecQ helicases may be structurally designed and enzymatically suited to act on three- and four-stranded DNA replication and recombination intermediates. In addition to unwinding and annealing activity, WRN is the only human RecQ homologue that also possesses a 3′ to 5′ exonuclease activity with similar substrate specificity for complex DNA structures as described above.[15−17]

A significant threat to genome stability is blockage of DNA synthesis that occurs when replication forks encounter DNA

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damage, abnormal DNA structures, or proteins bound to parental DNA. To ensure survival subsequent to such encounters, cells have evolved pathways to deal with replication fork blockage that facilitate replication restart and completion of normal DNA synthesis. RecQ helicases, including WRN and BLM, are commonly postulated to act in pathways that overcome replication fork blockage.[18−20] Consistent with this hypothesis, WRN-deficient cells are hypersensitive to replication blocking agents, including hydroxyurea (HU), topoisomerase inhibitors, and interstrand cross-linking agents such as mitomycin C, grow slowly in culture with an extended S-phase, and exhibit specific replication abnormalities, including asymmetry in normal bidirectional replication fork progression.[21−24] Furthermore, in normal cells, with the use of DNA-damaging treatments with HU or other agents, WRN is recruited to sites of blocked replication and colocalizes with other replication factors in distinct nuclear foci.[25−27] Similarly, BLM-deficient cells are also hypersensitive to mitomycin C, hydroxyurea, and aphidicolin and exhibit replication abnormalities, including problems in restart of replication forks after HU and aphidicolin treatment.[28−32] Following DNA-damaging treatment with HU or the interstrand cross-linking agent psoralen, BLM also relocalizes to sites of ongoing replication that contain PCNA and other replication factors.[33,34] Collectively, this evidence suggests that both WRN and BLM may be involved in pathways responding to replication blockage.

According to some models, an initial step in dealing with a blocked replication fork may be fork regression to generate a Holliday junction or "chicken foot" intermediate.[20,35−37] Following fork regression, the block to replication may be overcome by (1) repair of the DNA lesion and reverse branch migration to re-establish a fork structure, (2) use of an extended lagging daughter strand as a template for the synthesis of the leading strand followed by reverse branch migration to bypass the blocking lesion to re-establish the fork, or (3) use of the free double-stranded end of this Holliday junction to initiate RAD51-mediated strand invasion and re-establishment of a functional replication fork. Alternatively, processing or resolution of the Holliday junction could generate a double-strand break that also could be utilized by RAD51-mediated recombinational pathways to restore a functional replication fork. In any case, a coordinated, error-free mechanism for resolution of replication blockage and re-establishment of the replication fork is absolutely critical for maintaining genome stability and promoting cell survival.

As both WRN and BLM bind to and branch migrate Holliday junctions in vitro,[25,38−40] each could potentially convert a regressed fork back to a functional replication fork. To determine if WRN and/or BLM might catalyze this important step in resolution of replication blockage, we constructed a specialized Holliday junction structure and investigated whether WRN or BLM could convert it to a four-stranded fork structure that could subsequently be subject to DNA replication. Our results demonstrate that both WRN and BLM efficiently catalyze conversion of this Holliday junction to a replication fork structure, with its leading daughter strand capable of being extended in vitro by DNA polymerases. Consistent with the idea that WRN and BLM target the Holliday junction structure to initiate branch migration, this conversion reaction was inhibited by RuvA and more efficient with WRN and BLM compared to the known branch migration enzyme RAD54. Also, WRN (and BLM) exhibits enhanced binding to and action on this Holliday junction substrate at low Mg^{2+} concentrations, suggesting they preferentially act on the square planar conformation of Holliday junctions. Our results suggest that WRN and/or BLM might act to reset the replication fork subsequent to fork stalling and regression, functions that are highly consistent with the replication abnormalities and specific genomic instability phenotype(s) associated with WS and BS.

EXPERIMENTAL PROCEDURES

Enzymes. All recombinant human WRN proteins (WRN-wt, WRN-E84A, and WRN-K577M) used in these studies were overexpressed in insect cells and purified as previously described.[41] WRN-E84A contains a glutamate to alanine mutation that abolishes its 3′ to 5′ exonuclease activity but preserves its normal DNA-dependent ATPase, helicase, and annealing activities; WRN-K577M contains a lysine to methionine mutation that abolishes ATPase and helicase activity but retains exonuclease activity.[14,16,42] To generate mock preparations, insect cells infected with baculovirus without WRN sequences were lysed and subjected to purification procedures identical to those used for WRN proteins. Recombinant human RECQ4 was purified as described previously.[8] Human wild-type BLM and BLM-D795A protein containing an aspartate to alanine mutation that abolishes its ATPase and helicase activities were gifts from J. Groden (The Ohio State University, Columbus, OH), while human RPA and human (four-subunit) DNA polymerase δ were gifts from G.-M. Li (University of Kentucky). Purified *Escherichia coli* UvrD and human RAD54 were obtained from S. Matson (University of North Carolina, Chapel Hill, NC) and A. Mazin (Drexel University, Philadelphia, PA), respectively. Purified *E. coli* RuvA was purchased from B-Bridge International. Klenow fragment (3′ to 5′ exo$^-$), from New England Biolabs, is an N-terminal truncation of *E. coli* DNA polymerase I that lacks both 5′ to 3′ and 3′ to 5′ exonuclease activities but retains polymerase activity. T4 DNA polymerase and T4 polynucleotide kinase were purchased from New England Biolabs. Proteinase K was obtained from Invitrogen.

DNA Substrate Construction. The sequences of oligonucleotides (purchased from Integrated DNA Technologies) used in this study are listed in Table 1 of the Supporting Information. LeadD81, LagP70, and 3-way-base62 were radiolabeled (indicated by asterisk hereafter and in figures) at their 5′ ends by standard procedures using [γ-^{32}P]ATP and T4 polynucleotide kinase, and unincorporated nucleotide was removed using a spin column (Roche Applied Science). Unlabeled LagD84 was annealed to *LeadD81 by being heated to 95 °C for 5 min and slowly cooled; in parallel, unlabeled LeadP122 and LagP122 were similarly annealed. The resulting two-stranded fork structures were annealed to each other for 16 h at 25 °C to generate a singly labeled, static Holliday junction structure (see Figure 1A, left). To generate the four-stranded replication fork substrate, the heating and slow cooling procedure was used to anneal (1) *LeadD81 with unlabeled LeadP122 and (2) unlabeled LagD84 with unlabeled LagP122; subsequently, the resulting partial duplexes were annealed for 16 h at 25 °C. Two-stranded fork (*LeadD81/LagD84 and *3-way-base62/3-way-5′flap), partial duplex (*LeadD81/LeadP122 and *LagP70/LagD30), and three-way junction (*3-way-base62/3-way-3′flap/3-way-5′flap) DNA substrates were generated by annealing the relevant oligonucleotides using the heating and slow cooling procedure. After formation,

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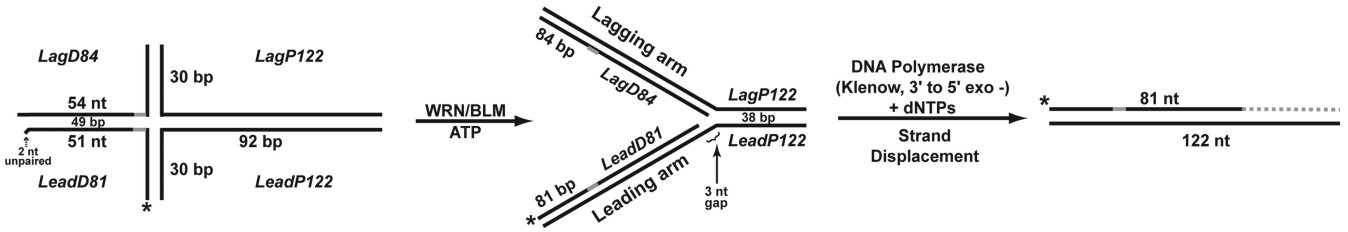

Figure 1. Diagram of Holliday junction substrate structure, conversion, and extension products. Schematic showing putative WRN- or BLM-mediated conversion of our specialized Holliday junction structure (left) to a replication fork (center) with subsequent DNA polymerase-mediated extension from the 3′ end of the labeled (denoted with an asterisk) leading daughter strand of the fork. Most experiments utilized Klenow fragment, 3′ to 5′ exo$^-$ as DNA polymerase, resulting in strand displacement and new DNA synthesis (denoted with a dotted gray line) to the end of the parental leading strand template (right). While the vertical arms of this Holliday junction substrate are nonhomologous, the left arm is completely homologous to the proximal 51−54 bp of the right arm except for 5 bp of nonhomology (denoted with a solid gray segment) at the junction. This design ensured a static Holliday junction structure that could be converted to a replication fork structure (by enzymatically branch migrating the junction rightward, overcoming the short nonhomologous segment). Note that in the context of the Holliday junction, two nucleotides at the 3′ end of the labeled strand were unpaired; upon conversion to replication fork structure, these nucleotides are fully base-paired. Names of the oligos used for substrate construction are italicized; nucleotide sequences for these oligos are given in Table 1 of the Supporting Information.

all substrates were purified by native polyacrylamide (6%) gel electrophoresis, excised from the gel after autoradiography, eluted for 24 h into buffer containing 10 mM Tris (pH 8.0) and 10 mM NaCl, and stored at 4 °C.

Enzymatic Assays. For studying the conversion of the Holliday junction to fork DNA, Holliday junction substrate (~2 fmol) was incubated for the specified times at 37 °C with WRN-E84A, WRN-wt, WRN-K577M, BLM, BLM-D795A, RAD54, RECQ4, or UvrD (at concentrations indicated in the figure legends) in WRN reaction buffer (20 μL) containing 40 mM Tris-HCl (pH 8.0), 0.1 mg/mL BSA, 5 mM dithiothreitol, and 0.1% Nonidet P-40 supplemented with $MgCl_2$ (0.5−8 mM, as indicated) and either ATP (0.125−2 mM) or ATPγS (1 mM). Prior to incubation at 37 °C in specific experiments, (1) RuvA (4−1000 fmol) was preincubated with Holliday junction substrate for 5 min at 4 °C before addition of WRN-E84A or BLM, or (2) RPA (2.5−80 fmol) was added subsequent to WRN-E84A while the reaction mixtures were kept at 4 °C. Unwinding assays using two-stranded fork and partial duplex substrates were performed similarly; however, assays with RECQ4 and three-way junction substrate also contained a 25-fold excess of unlabeled 3-way-base62 oligomer. Reactions were terminated by the addition of 5 mM EDTA; then, 10 μg of proteinase K was added, and the samples were incubated at 37 °C for 10 min. Following addition of $^1/_6$ volume of loading dye (30% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol), samples were subjected to native polyacrylamide (5−6%) gel electrophoresis (PAGE) in 1× TBM [90 mM Tris-borate (pH 8.0) and 5 mM $MgCl_2$] at 125 V for 3.5 h. The gels were dried, and the radioactivity associated with the DNA products was visualized and quantitated by phosphorimaging analysis (Storm 860 phosphorimager and ImageQuant, GE Healthcare). Conversion of Holliday junction to four-stranded replication fork was measured as a percentage of the radioactivity associated with replication fork to that of the total DNA, after subtraction of low background levels of DNA species present in control reaction mixtures without enzyme. Similarly, substrate unwinding was quantified as the percentage of radioactivity associated with the unwound product with respect to the total DNA in the reaction, again correcting for any background level of the product species in reactions without enzyme. To assess the 3′ to 5′ exonuclease activity inherent in WRN-wt and WRN-K577M, DNA products from reactions performed as described above were separated by denaturing PAGE (10%); after gel drying, exonucleolytic degradation from the 5′ end-labeled *LeadD81 strand of the substrate and/or products was visualized by phosphorimaging.

Assays were also performed to study the action of DNA polymerases on the conversion product as well as various control substrates. For these studies, Holliday junction, four-stranded replication fork, and two-stranded fork (*LeadD81/LagD84) substrates (2−4 fmol) were incubated with or without either WRN-E84A, a mock preparation of WRN, BLM, or BLM-D795A (at concentrations specified in the figure legends) in WRN reaction buffer (20 μL) containing 4 mM $MgCl_2$, either 1 mM ATP or 1 mM ATPγS, and 100 μM dNTPs. Following incubation at 37 °C, Klenow (3′ to 5′ exo$^-$) (0.01−0.001 unit/reaction), T4 DNA polymerase (0.005 unit), or human DNA polymerase δ (60 fmol) was added with further incubation at 37 °C (times indicated in figure legends). These reactions were stopped by withdrawing a 10 μL aliquot from the reaction mixture and adding an equal volume of formamide loading buffer (95% formamide, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol). The DNA products were subsequently heated at 90 °C for 5 min and separated by denaturing PAGE (6%) in 1× TBE at 40−45 W for 1.5 h. The gels were dried and analyzed by phosphorimaging as described above. The percentage of extension (by Klenow) of the labeled leading daughter strand (of four-stranded forks converted from Holliday junctions) was calculated by dividing the combined signal for all products with higher molecular weights (compared to that of the original unextended strand) by the total radioactive signal from the entire lane (amount of unextended leading daughter strand + combined extended products).

Electrophoretic Mobility Shift Assay (EMSA). Holliday junction substrate (~4 fmol) was incubated with WRN-E84A (0−12 fmol) in WRN reaction buffer (20 μL) supplemented with either 1 or 8 mM $MgCl_2$ for 10 min at 25 °C. Subsequently, $^1/_6$ volume of 30% glycerol was added and samples were separated by native PAGE (3.5%, acrylamide:bisacrylamide cross-linking ratio of 37.5:1) in 0.5× TBE at 100 V for 2 h. The gels were dried and subjected to phosphorimaging analysis as described above. WRN-E84A binding was assessed by comparing the amount of bound DNA with the total amount of DNA for each reaction [% DNA bound = (DNA bound/total DNA) × 100].

RESULTS

Specialized Holliday Junction Substrate Design. It has been postulated that, as part of a pathway to overcome replication blockage, some RecQ helicases, including WRN and BLM, may reset the replication fork from Holliday junctions (or chicken foot structures) generated by fork regression.[18,20] To examine a potential role for WRN or BLM in re-establishing a functional replication fork following regression, we constructed a special Holliday junction substrate (Figure 1, left) using a two-step process: independently annealed *LeadD81/LagD84 and LeadP122/LagP122 species were subsequently annealed to one another. The resulting Holliday junction substrate contained two 30 bp arms (pictured vertically in Figure 1) that were completely nonhomologous; the left arm was completely homologous to the proximal 51−54 bp of the right arm, except for five nonhomologous base pairs at the junction. As constructed, this structure was static but could potentially be converted to a four-stranded replication fork (Figure 1, center) by branch migration of the Holliday junction (rightward in this orientation) through the region of nonhomology. In the event this conversion occurred, this replication fork structure would contain a three-nucleotide single-stranded gap on the leading arm at the fork junction. In the original Holliday junction substrate, the strand that would become the leading daughter strand following conversion to fork was labeled so that DNA polymerase-mediated extension from its 3′ end could be monitored. In the context of the Holliday junction structure, the last two nucleotides on the 3′ end of this strand were unpaired; this ensured that this strand could not be extended by a DNA polymerase unless conversion occurred (or without removal of the unpaired nucleotides by a 3′ to 5′ exonuclease). The design of this substrate allowed us to monitor enzyme-mediated conversion to the four-stranded replication fork structure by assaying (1) a structure-dependent change in migration by native PAGE and (2) extension from the 3′ end of the labeled strand by DNA polymerases, analyzed by denaturing PAGE. Notably, strand displacement activity inherent in Klenow fragment (and, to a lesser degree, in human DNA polymerase δ) allowed polymerization of the labeled leading daughter strand of the four-stranded fork structure (Figure 1, center) beyond the fork junction to the end of the leading parental strand template to generate labeled extension products of up to 122−123 nucleotides (Figure 1, right). The following sections detail how purified WRN and BLM proteins act on this specialized Holliday junction substrate.

WRN and BLM Convert Holliday Junction Substrate to Four-Stranded Replication Fork. To test the hypothesis that RecQ helicases may be able to act on a Holliday junction structure reflecting a regressed fork and re-establish a functional replication fork, our Holliday junction substrate was incubated with or without purified enzymes. For most WRN-containing reaction mixtures, the exonuclease-deficient WRN-E84A mutant was used so that the reaction products could be analyzed without complication from possible digestion of the substrate or the products; importantly, use of this mutant also eliminated the possibility that the 3′ to 5′ exonuclease activity of WRN could remove the two unpaired nucleotides on the 3′ end of the labeled strand of the original Holliday junction substrate. Under standard conditions of electrophoresis using native PAGE with 1× TBE as the running buffer, the mobilities of the Holliday junction substrate and the four-stranded fork DNA marker representing the conversion product were quite similar. Because it has been shown previously that the mobility of Holliday junctions in polyacrylamide gels is influenced by divalent cation concentration,[43,44] we included 5 mM $MgCl_2$ (and omitted EDTA) in both our gels and running buffers. Under these conditions, we obtained adequate separation between the Holliday junction and the four-stranded fork DNA, the former migrating substantially slower than the latter. Theoretically, unwinding of a four-stranded Holliday junction by a helicase could yield a number of one-, two-, or three-stranded products; in fact, WRN and BLM have been previously shown to unwind partly mobile Holliday junction substrates to generate partial duplex as well as single-stranded products.[39,40] However, when we incubated our specialized Holliday junction substrate with either WRN-E84A or BLM in the presence of 4 mM $MgCl_2$ and 1 mM ATP, the primary product generated over time was the four-stranded replication fork structure (Figure 2A−D); little or no further unwinding of this fork to a partial duplex (*LeadD81/LeadP122) was detected. In addition to the conversion of Holliday junction to four-stranded fork, some unwinding of the Holliday junction to form the two-stranded *LeadD81/LagD84 fork species was also observed in these reactions (Figure 2A,B). Conversion of Holliday junction to four-stranded fork by both WRN-E84A and BLM was direct; i.e., formation of the four-stranded fork did not result from separate unwinding and annealing steps. Note that the *LeadD81/LagD84 species could not be a relevant intermediate in fork formation and no significant amounts of other products were detected at early time points in kinetic assays (Figure 2A,B). Notably, this conversion reaction requires ATP hydrolysis, as fork formation was not detected in reaction mixtures lacking ATP or containing ATPγS, a nonhydrolyzable analogue of ATP (Figure 2E,F). Furthermore, use of ATPase- and helicase-deficient BLM-D795A or WRN-K577M mutant proteins in these reactions also did not cause any detectable conversion of Holliday junction to fork product (Figure 2F and Figure 1 of the Supporting Information), confirming that these ATPase- and helicase-dependent conversion reactions are specifically catalyzed by BLM and WRN. Notably, the Holliday junction substrate is subjected to degradation by the 3′ to 5′ exonuclease activity retained in WRN-K577M (Figure 1 of the Supporting Information, bottom panel).

The precise structure of Holliday junctions is known to be variable depending on Mg^{2+} ion concentration, with a stacked X-structure becoming favored over a square planar structure as the Mg^{2+} concentration increases.[43,44] Because our results and previous reports show that WRN and BLM readily act on Holliday junction structures, it is highly relevant to also determine the effect of $MgCl_2$ concentration on WRN- and BLM-mediated conversion of our Holliday junction to replication fork product, keeping in mind that Mg^{2+} is a required cofactor in these reactions. In the presence of a fixed amount of ATP (1 mM), the amount of conversion of a Holliday junction to fork product by WRN-E84A was highly dependent on the $MgCl_2$ concentration (Figure 2G), being optimal between 0.5 (the minimal concentration tested) and 2 mM $MgCl_2$. The level of WRN-E84A-mediated conversion declined sharply at 4 mM $MgCl_2$ and even more drastically at 8 mM $MgCl_2$. To determine whether it is the absolute concentration of $MgCl_2$ or the $MgCl_2$:ATP ratio in the reaction mixture that influences this activity, reactions were set up in which ATP concentration was varied over a wide range (0.125−2 mM) in the presence of 1 mM $MgCl_2$, an optimal concentration in the experiment described above.

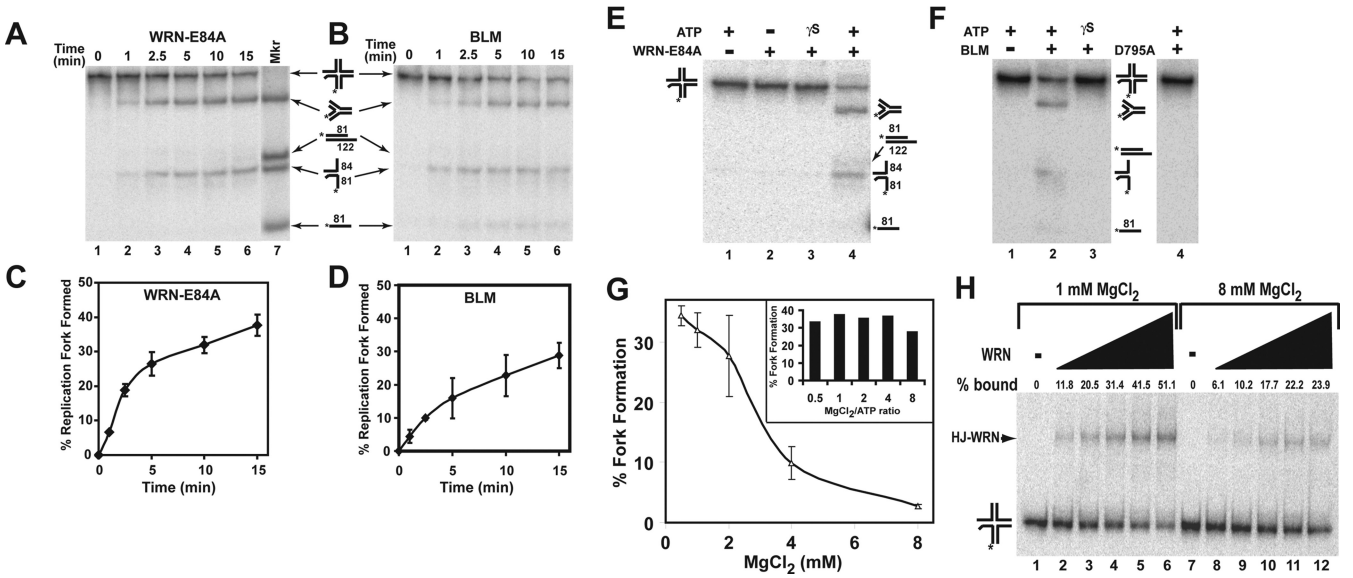

Figure 2. ATP and Mg^{2+} dependence of the direct conversion of specialized Holliday junction to a replication fork by WRN-E84A and BLM. Holliday junction substrate (~2 fmol) was incubated with (A) WRN-E84A (18 fmol) or (B) BLM (12.5 fmol) in $MgCl_2$ (4 mM) and ATP (1 mM) at 37 °C for the indicated times. DNA products (lanes 1−6) and marker substrates (lane 7, four-stranded replication fork, *LeadD81/LeadP122 partial duplex, *LeadD81/LagD84 fork, and *LeadD81 oligomer, migration positions depicted at the right) were analyzed by native PAGE as described in Experimental Procedures. Relative positions of migration of these marker substrates are applicable for panels B, E, and F and elsewhere. (C) Percentages of replication fork formation (calculated as described in Experimental Procedures) mediated by WRN-E84A plotted vs time for ATP-containing reactions conducted as described for panel A. Data are the mean and SE of three independent experiments. (D) Data from experiments performed as described for panel B were calculated and presented as described for panel C. (E) Holliday junction substrate (~2 fmol) was incubated for 30 min at 37 °C with WRN-E84A (15 fmol) with or without ATP or ATPγS (1 mM) as indicated, and the DNA products were analyzed by native PAGE as described for panel A. Positions of migration of specific DNA structures are depicted here at right and also in panel F. (F) Holliday junction substrate (~2 fmol) was incubated with BLM (12.5 fmol) or BLM-D795A (ATPase- and helicase-deficient mutant) (37.5 fmol) with ATP or ATPγS (1 mM) as indicated and incubated at 37 °C for 30 min, and then analyzed as described for panel A. (G) Holliday junction substrate (~2 fmol) was incubated with WRN-E84A (18 fmol) in the presence of ATP (1 mM) and varying concentrations of $MgCl_2$ (0.5, 1, 2, 4, and 8 mM) for 30 min at 37 °C, and the reaction products were analyzed by native PAGE as described in the legend of Figure 1. For three independent experiments, the mean percentage (±standard deviation) of conversion of Holliday junction to fork structure is plotted vs $MgCl_2$ concentration. In the inset of panel G, similarly, reaction mixtures containing Holliday junction substrate (~2 fmol), WRN-E84A (18 fmol), 1 mM $MgCl_2$, and varying concentrations of ATP (0.125, 0.25, 0.5, 1, and 2 mM) were incubated for 30 min at 37 °C and the percentage of conversion to fork structure is depicted for each $MgCl_2$:ATP ratio. (H) Holliday junction substrate (4 fmol) was incubated with WRN-E84A (0, 1.5, 3, 6, 9, and 12 fmol) in the absence of ATP and in the presence of either 1 or 8 mM $MgCl_2$ as indicated for 10 min at 25 °C, and protein−DNA complexes were resolved from free DNA by an EMSA as described in Experimental Procedures. The percentage of Holliday junction bound by WRN-E84A is calculated as described in Experimental Procedures and denoted above each lane. Positions of free Holliday junction substrate and Holliday junction−protein (HJ−WRN) complexes are noted at the left.

Thus, the $MgCl_2$:ATP ratio in these reaction mixtures varied between 0.5 and 8. The results of this experiment (Figure 2G, inset) reveal no substantial difference in the conversion of Holliday junction to fork DNA by WRN-E84A over the range of $MgCl_2$:ATP ratios tested, with conversion percentages ranging between 28 and 38%. Given that the inhibitory effect of increasing the $MgCl_2$ concentration from 2 mM to either 4 or 8 mM on fork formation was much stronger, our results suggest that a larger absolute amount of Mg^{2+} was inhibitory with respect to the WRN-mediated conversion process. In contrast, unwinding of a 3′ overhang partial duplex by WRN-E84A was not significantly inhibited by $MgCl_2$ concentrations up to 4 mM (Figure 2 of the Supporting Information), suggesting that the marked inhibition of the Holliday junction conversion reaction at Mg^{2+} concentrations of ≥4 mM was not due to general loss of WRN catalytic activity. The effect of $MgCl_2$ concentration on BLM-mediated conversion followed the same general pattern as that of WRN-E84A, although the inhibitory effect at 4 and 8 mM $MgCl_2$ was much less pronounced compared to that on WRN-E84A (Figure 3A of the Supporting Information). These findings are consistent with an effect of a higher Mg^{2+} concentration on the structure of the Holliday junction substrate that weakens the ability of WRN-E84A and BLM to catalyze the conversion reaction.

To further explore whether the nature of Holliday junction structure influenced enzyme function, we used EMSA to determine if there was any difference in the binding affinity of WRN-E84A for the Holliday junction substrate at low (1 mM) and high (8 mM) $MgCl_2$ concentrations. It has been shown previously that some RecQ helicases, including WRN and BLM, exhibit high-affinity binding and enhanced catalytic activities on three- and four-stranded DNA structures, including Holliday junctions and forks as compared to single-stranded and duplex DNA.[7,39,45,46] When binding reactions were performed in the presence of 1 mM $MgCl_2$, WRN-E84A formed stable and specific complexes with Holliday junction substrate as observed after EMSA (Figure 2H). The amount of enzyme−DNA complex was dependent on WRN-E84A, with >50% of the substrate bound at the highest concentration. Although discrete and stable binding was observed between WRN-E84A

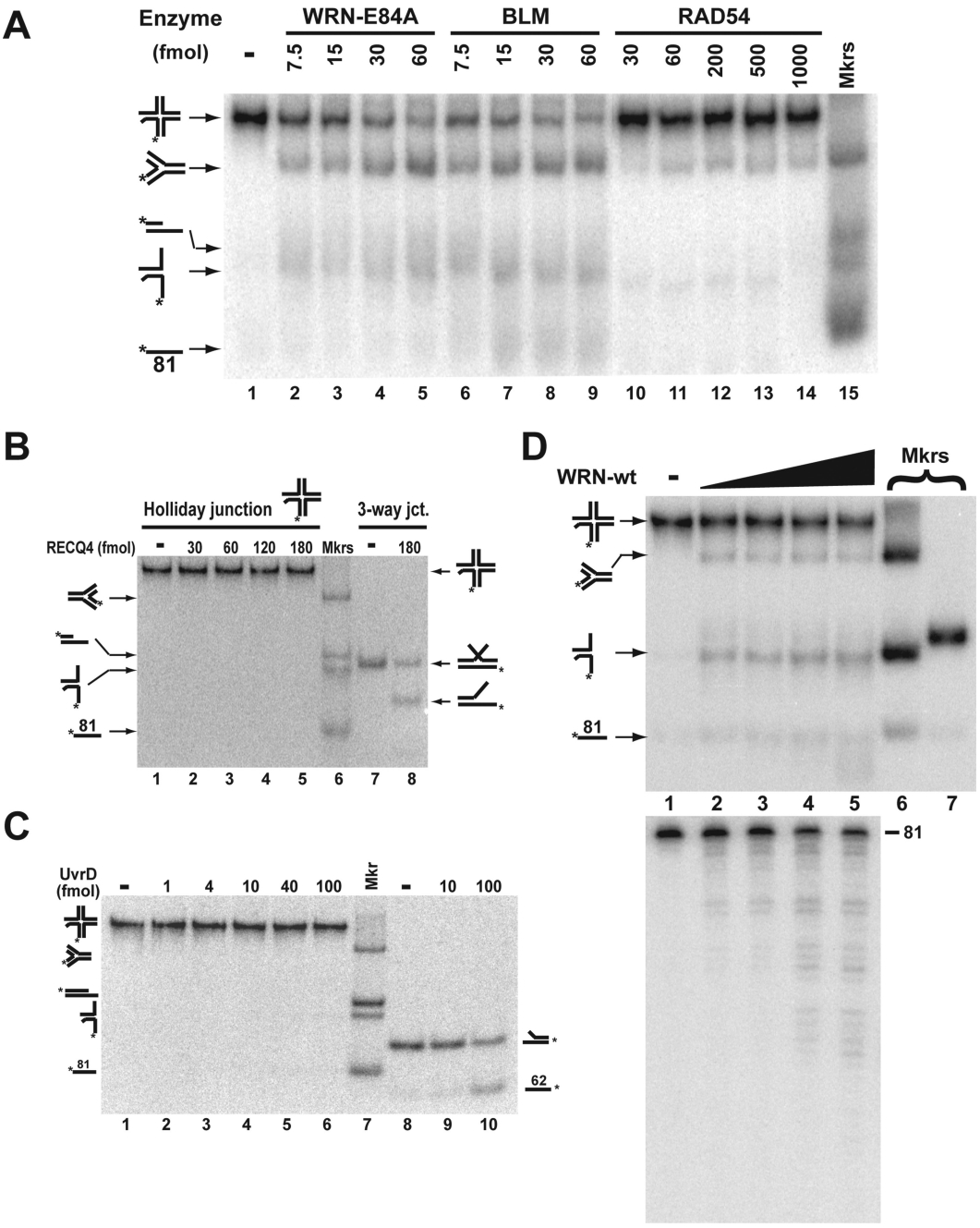

Figure 3. WRN and BLM preferentially convert the Holliday junction to replication fork. (A) Holliday junction substrate (~2 fmol) was incubated with WRN-E84A, BLM, or RAD54 at the indicated concentrations for 30 min at 37 °C in 1 mM $MgCl_2$ and 1 mM ATP. DNA products of these reactions (lanes 1−14) and markers (lane 15) were analyzed as described in the legend of Figure 2A. (B) Holliday junction (~2 fmol) or three-way junction substrate (5 fmol, plus 125 fmol of unlabeled 3-way-base62 trap strand) was incubated with human RECQ4 at the indicated concentrations for 30 min at 37 °C in 4 mM $MgCl_2$ and 1 mM ATP. DNA products and markers were analyzed as described for panel A. (C) At the indicated concentrations, UvrD was incubated with either 2 fmol of Holliday junction substrate (lanes 1−6) or 5 fmol of two-stranded fork (*3-way-base62/3-way-5′flap) substrate (lanes 8−10) for 30 min at 37 °C. Specific preformed DNA structures were loaded as markers (lane 7) with their positions of migration depicted on the left and right sides. (D) Holliday junction (~2 fmol) was incubated without or with WRN-wt (7.5, 15, 30, and 45 fmol) for 30 min at 37 °C in 1 mM $MgCl_2$ and 1 mM ATP. DNA products of these reactions (lanes 1−5) were analyzed both by native PAGE (top) and by denaturing PAGE (bottom). Also loaded on native PAGE were markers for replication fork and *LeadD81/LagD84 fork species (lane 6) and for the *LeadD81/LeadP122 partial duplex species (lane 7).

and Holliday junction DNA when binding reaction mixtures contained 8 mM $MgCl_2$, the level of binding was significantly reduced at each concentration of WRN-E84A, with <25% of the original DNA stably bound at the highest concentration (Figure 2H). This clearly demonstrates that the initial binding of WRN to Holliday junction substrate or stability of the protein−DNA complexes is higher at 1 mM than at 8 mM $MgCl_2$. Taken together, our results indicate that increasing Mg^{2+} concentrations (>2 mM) inhibit the binding and conversion activity of WRN on our Holliday junction substrate. Furthermore, these findings suggest that these enzymes preferentially bind to and act on the square planar

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conformation of Holliday junctions compared to the stacked X form.

Specificity of the WRN- and BLM-Mediated Conversion Reaction. Substantial evidence implicates both WRN and BLM helicases in the response to replication fork blockage and replication restart. Biochemically, each enzyme has previously been demonstrated to have not only helicase activity but also branch migration activity.[25,38] Our conversion reaction, the re-establishment of a replication fork from a (regressed) Holliday junction, would seem to most closely reflect branch migration, and thus, it may shed light on the physiological function of these enzymes and perhaps other RecQ helicases. However, we wanted to determine the relative specificity and/or preference of WRN and BLM to catalyze this conversion in comparison with other helicases and branch migration enzymes. Human RAD54 has been demonstrated to branch migrate Holliday junctions and recently has been shown to convert a Holliday junction substrate to a replication fork structure, quite similar to our conversion reaction.[47,48] Therefore, we directly compared the actions of WRN-E84A, BLM, and RAD54 on our Holliday junction substrate. WRN-E84A and BLM catalyzed similar levels of conversion at equivalent protein concentrations, with the percentage of conversion (compared to the total amount of DNA in the reaction) reaching 42.3 and 36.6%, respectively, at the highest enzyme concentrations (60 fmol) tested (Figure 3A, lanes 2−9). Although the four-stranded replication fork was detectable when RAD54 was used, the level of conversion was very limited (a maximum of 10.2%) even at much higher protein concentrations (Figure 3A, lanes 10−14). It is also notable that RAD54 could not detectably catalyze this conversion reaction when a higher $MgCl_2$ concentration (4 mM) was used (Figure 3B of the Supporting Information). We also tested another RecQ helicase, human RECQ4, and a typical bacterial helicase, *E. coli* UvrD, in our conversion reaction. Interestingly, RECQ4 unwinding appears to require an "extra" complementary strand,[8] suggesting it may catalyze a strand exchange type of reaction instead of typical unwinding. Indeed, we were able to confirm this type of unwinding/strand exchange activity in RECQ4 when using a three-way junction substrate with a 25-fold excess of complementary strand (Figure 3B, lane 8). However, RECQ4 had no detectable activity on our Holliday junction substrate (Figure 3B, lanes 2−5). Similarly, UvrD did not facilitate formation of the fork product or any other DNA species from our Holliday junction substrate over a wide concentration range (Figure 3C, lanes 2−6), although this helicase could readily unwind a two-stranded fork substrate (Figure 3C, lanes 9 and 10). Taken together, our results indicate that the conversion of our Holliday junction substrate to a four-stranded replication fork involves branch migration activity as opposed to simple unwinding or strand exchange activity. Perhaps more importantly, WRN and BLM preferentially catalyze this specific reaction in comparison to another branch migration enzyme, human RAD54, while this activity is completely lacking in another human RecQ helicase, RECQ4.

WRN is unique among the human RecQ helicases in that it also possesses an inherent 3′ to 5′ exonuclease, the physiological function of which remains unclear. However, previous reports have shown that WRN's exonuclease activity is highly active on 3′ ends proximal to unusual DNA structures, including forks and bubbles.[17,42,49] Thus, we investigated the processing of our specialized Holliday junction substrate by WRN-wt, using native PAGE to analyze the overall effect on the substrate and denaturing PAGE to assess exonucleolytic degradation from the 3′ end of the labeled strand. When the substrate was incubated with WRN-wt (Figure 3D, top panel), again the four-stranded replication fork product was generated, although present in seemingly smaller amounts than for WRN-E84A. Also, as compared with that of WRN-E84A, higher levels of products apparently stemming from *LeadD81/LagD84 fork and single-stranded *LeadD81 were noted, suggestive of significant unwinding. Examination of the same reactions by denaturing PAGE showed substantial digestion from the 3′ end of the radiolabeled strand (Figure 3D, bottom panel), indicating that WRN's 3′ to 5′ exonuclease activity could readily target the reaction substrate and/or products. Upon close examination, WRN-mediated exonucleolytic degradation is also noticeable on native PAGE, as evidenced by the slightly faster migration of the two-stranded fork product than its undigested *LeadD81/LagD84 marker and the faint smear of labeled single-stranded products downward compared to the position of the single-stranded *LeadD81 marker (Figure 3D, top panel). Importantly, denaturing PAGE cannot reveal degradation of the unlabeled strands of the substrate or products, and it seems clear that the substrate and products are structurally altered by WRN exonuclease activity. The notion that WRN's exonuclease activity can alter the original Holliday junction structure is confirmed by the 3′ to 5′ digestion of the labeled strand observed in the presence of WRN-K577M, the ATPase- and helicase-deficient mutant that cannot unwind or branch migrate the substrate (Figure 1 of the Supporting Information, bottom panel). Given that the substrate and product structures are in flux during the course of the reaction due to WRN exonuclease activity, it becomes very difficult to accurately assess how the combined helicase and exonuclease activities process this substrate. Nevertheless, it can be concluded that, in the context of the Holliday junction/chicken foot structure, the 3′ end of the leading daughter strand is readily accessible to WRN exonuclease function. We can also conclude that, despite ongoing WRN exonuclease-mediated digestion of the Holliday junction substrate, WRN-wt protein converts a substantial amount of this substrate to four-stranded replication fork.

Effects of RuvA and RPA on WRN- and BLM-Mediated Conversion of Holliday Junction to Replication Fork. The WRN- and BLM-mediated conversion reaction described above suggests that each enzyme can specifically bind to the Holliday junction structure and initiate branch migration to result in replication fork formation. To determine whether WRN and BLM specifically target the Holliday junction, we tested the effect of *E. coli* RuvA, a Holliday junction-specific binding protein without catalytic activity, on our conversion reactions. In these experiments, RuvA was preincubated with the Holliday junction substrate before either WRN-E84A or BLM was added to initiate the reaction; as in standard unwinding/conversion assays, the DNA products were examined by native PAGE after removal of the proteins. As expected, RuvA alone had no effect on the Holliday junction substrate (Figure 4, lanes 14 and 15). As previously observed, WRN-E84A alone primarily catalyzed conversion of the Holliday junction to four-stranded replication fork, as did BLM alone (Figure 4, lanes 2 and 8). Even at the lowest concentration tested, RuvA drastically reduced WRN-mediated production of the replication fork and further inhibited this reaction when higher levels of RuvA were employed (Figure 4, lanes 3−7). Surprisingly, the effect of RuvA in reaction mixtures

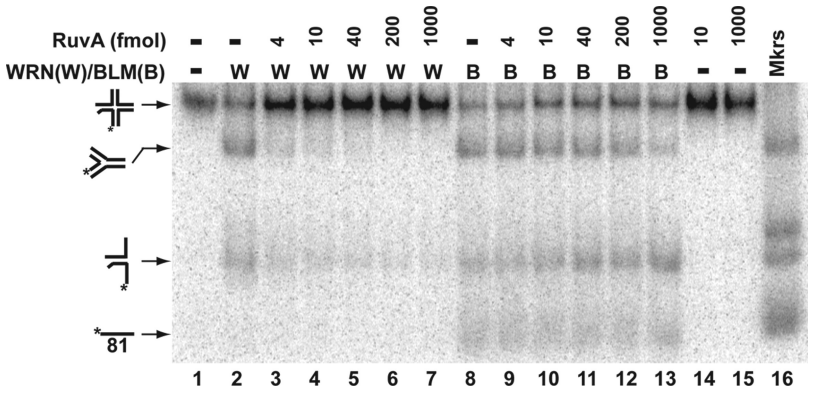

Figure 4. RuvA inhibits WRN- and BLM-mediated conversion of Holliday junction to replication fork. Holliday junction substrate (2 fmol) was preincubated with RuvA (0−1000 fmol, as indicated) in WRN reaction buffer containing 1 mM $MgCl_2$ and 1 mM ATP at 4 °C for 5 min; where indicated, WRN-E84A (30 fmol) or BLM (12 fmol) was added, and the reaction mixtures were incubated for 30 min at 37 °C. The DNA products (lanes 1−9), along with markers for four-stranded replication fork, *LeadD81/LeadP122, and *LeadD81/LagD84 (lane 10), were separated by native PAGE and visualized as described in Experimental Procedures.

containing BLM was more modest, with substantial inhibition of BLM-mediated conversion only observed at the highest RuvA concentrations (Figure 4, lanes 9−13). These results suggest that RuvA binding to the Holliday junction essentially blocks WRN from accessing the substrate to catalyze conversion, but somehow BLM is able to almost completely override RuvA's effect.

Replication protein A (RPA) has been postulated to play an important role in the response to replication blockage and has also been shown to modulate WRN and BLM activities.[25,50−53] It is therefore relevant to determine whether RPA influences the conversion of Holliday junction to replication fork. To this end, our specialized Holliday junction substrate was incubated with WRN-E84A or BLM in the absence or presence of increasing concentrations of human RPA. RPA alone had no effect on the substrate (Figure 4A,B of the Supporting Information, lanes 9−11). As before, either WRN-E84A or BLM alone primarily generated the four-stranded replication fork (Figure 4A,B of the Supporting Information, lane 2) Addition of RPA to reaction mixtures containing WRN-E84A or BLM had an only very minor effect on these reactions. Specifically, the primary product of both WRN-E84A- and BLM reactions remained the four-stranded replication fork with little or no change in its abundance or that of the *LeadD81/LagD84 unwinding product, even at RPA levels that were in 40-fold excess with respect to the level of the substrate (Figure 4A,B of the Supporting Information, lanes 3−8). In the WRN-E84A reactions, perhaps a very minor amount of the *LeadD81/LeadP122 partial duplex product is barely detectable at the highest RPA concentrations, probably a result of (forward) unwinding of the converted four-stranded replication fork. In the BLM-containing reaction mixtures, a modest increase in the level of the single-stranded *LeadD81 product is observed at the highest RPA concentrations, potentially resulting from substrate and/or product unwinding. Taken together, our results suggest that, at least for this reaction, RPA does not significantly shift the reaction in favor of or against replication fork formation, nor does it significantly alter the preference of WRN-E84A or BLM to perform branch migration versus unwinding.

DNA Synthesis Subsequent to WRN- or BLM-Mediated Conversion of Holliday Junction Substrate. To be productive in the context of overcoming replication blockage, enzyme-mediated action on a regressed fork (Holliday junction/chicken foot) should re-establish a functional fork subject to replication by DNA polymerases. To investigate this possibility, we sequentially incubated the Holliday junction substrate with either WRN-E84A or BLM followed directly by various DNA polymerases, under conditions otherwise identical to those of the conversion reactions except for the inclusion of dNTPs. Potential polymerase-mediated extension of the labeled strand was monitored after the reaction products were heated at 90 °C and separated by denaturing PAGE. As mentioned above, there are two unpaired nucleotides at the 3′ end of the labeled strand in the context of the original Holliday junction substrate; these nucleotides are fully base-paired upon conversion to the four-stranded fork. Thus, for most reactions, the DNA polymerase used was Klenow fragment, 3′ to 5′ exo$^-$ (hereafter termed Klenow), because its lack of 3′ to 5′ exonuclease activity eliminated the possibility of extension from the unpaired 3′ end of the original Holliday junction substrate. This was experimentally confirmed in reaction mixtures containing only Holliday junction substrate and Klenow that showed no extension of the labeled strand (Figure 5A, lane 3, and Figure 5B, lane 2). As expected, extension was also not detectable when only WRN-E84A or BLM was present (Figure 5A, lane 2, and Figure 5B, lane 3). Only when Klenow was added to reaction mixtures treated with either WRN-E84A or BLM in the presence of ATP was extension of the labeled strand observed (Figure 5A,B, lane 4). In these reactions, the primary extension product was 122−123 nucleotides in length, indicating that Klenow had synthesized beyond the fork junction to the end of the template (LeadP122) strand (see Figure 1, right). The identical product was also observed in control reactions when Klenow alone was incubated with the four-stranded fork (Figure 5A,B, lane 10) or the *LeadD81/LeadP122 partial duplex (data not shown). This result indicated that the strand displacement property inherent in Klenow is mostly responsible for extension beyond the fork junction, with possible assistance from WRN-E84A or BLM unwinding activity. The shortest extension products observed may be due to pausing caused by the fork junction, while intermediate products are likely pause sites specific for Klenow. Notably, *LeadD81/LagD84, a minor unwinding product in the conversion reaction (Figure 2A,B,E,F) containing two unpaired nucleotides at the 3′ end of the labeled strand, could

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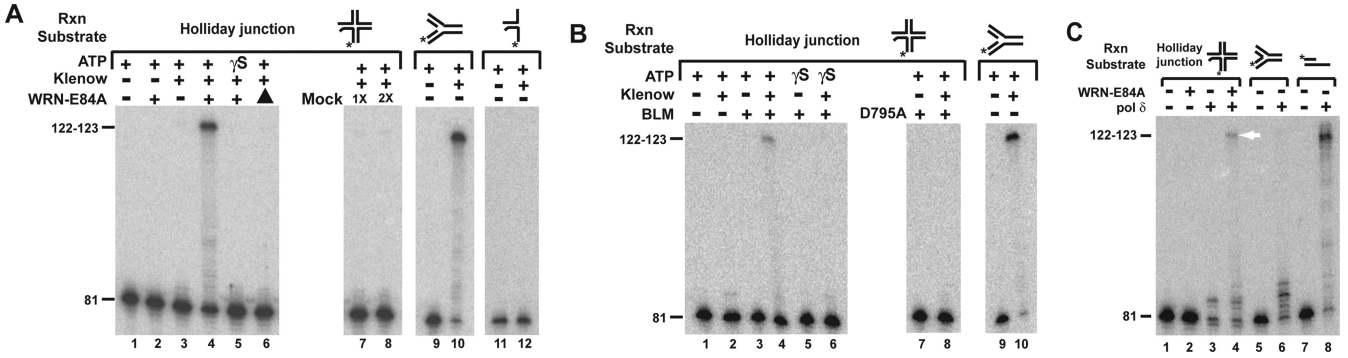

Figure 5. Extension of WRN- and BLM-converted Holliday junction substrate by Klenow (3′ to 5′ exo$^-$) and human DNA polymerase δ. (A) Holliday junction substrate (2 fmol) was incubated with either WRN-E84A (18 fmol) or mock-purified protein preparation (volume normalized, 1× and 2×) (lanes 7 and 8) in the presence of dNTPs (100 μM) and either ATP or ATPγS (1 mM) as specified for 10 min at 37 °C, followed by the addition of Klenow (3′ to 5′ exo$^-$) (0.001 unit) where indicated and further incubation at 37 °C for 20 min. In the reaction depicted in lane 6, WRN-E84A was heat-denatured at 65 °C for 5 min before its addition. Similarly, four-stranded fork substrate (2 fmol, lanes 9 and 10) and *LeadD81/LagD84 substrate (2 fmol, lanes 11 and 12) were also incubated without or with Klenow (3′ to 5′ exo$^-$) (0.001 or 0.002 unit, respectively) at 37 °C for 20 min in the presence of dNTPs (100 μM) and ATP (1 mM). Products were analyzed by denaturing PAGE as described in Experimental Procedures. (B) Holliday junction substrate (~2 fmol) was incubated without or with BLM (12.5 fmol) or BLM-D795A (37.5 fmol) in the presence of dNTPs (100 μM) and ATP or ATPγS (1 mM) for 10 min at 37 °C, followed by the addition of Klenow (3′ to 5′ exo$^-$) (0.001 unit) as indicated and further incubation at 37 °C for 20 min. Similar reaction mixtures containing ATP (1 mM), dNTPs (100 μM), and four-stranded fork substrate (2 fmol) without or with Klenow (3′ to 5′ exo$^-$) (0.001 unit) (lanes 9 and 10) were incubated for 20 min at 37 °C. Reaction products were analyzed as described for panel A. (C) Holliday junction substrate (2 fmol, lanes 1−4) was incubated with WRN-E84A (18 fmol) in the presence of ATP (1 mM) and dNTPs (100 μM) for 10 min at 37 °C, followed by the addition of human DNA polymerase δ (60 fmol) where indicated and further incubation at 37 °C for 20 min. Similarly, four-stranded fork substrate (2 fmol, lanes 5 and 6) and *LeadD81/LeadP122 substrate (2 fmol, lanes 7 and 8) were also incubated without or with polymerase δ (15 fmol) at 37 °C for 20 min in the presence of dNTPs (100 μM) and ATP (1 mM). Products were analyzed as described for panel A. The white arrow indicates the fully extended 122−123-nucleotide product only in the specific Holliday junction-containing reaction mixture incubated with WRN-E84A followed by polymerase δ.

not be extended by Klenow alone (Figure 5A, lanes 11 and 12). This result confirmed that all of the extension observed in our Holliday junction-containing reactions takes place on the converted four-stranded replication fork product. In control reaction mixtures containing Klenow, extension products were not observed when (1) ATPγS was substituted for ATP in the presence of WRN-E84A or BLM (Figure 5A, lane 5, and Figure 5B, lane 6), (2) WRN-E84A was heat-denatured prior to addition (Figure 5A, lane 6), (3) mock-WRN preparations were substituted for WRN-E84A (Figure 5A, lanes 7 and 8), or (4) ATPase- and helicase-deficient BLM-D795A was substituted for wild-type BLM (Figure 5B, lane 8). These findings demonstrate that conversion of our specialized Holliday junction substrate to a replication fork structure is a bona fide activity of WRN and BLM and indicate that ATPase-dependent conversion to four-stranded fork is required for subsequent extension of the labeled (leading daughter) strand by Klenow. It is notable that, although WRN can utilize dATP or dCTP hydrolysis to drive unwinding,[50] a 10-fold excess of ATPγS (1 mM) over dATP (0.1 mM) in these reactions is sufficient to completely inhibit conversion of Holliday junction to four-stranded fork. In WRN-containing reaction mixtures, approximately 50% of the labeled strand was extended (to any extent), suggesting that most if not all of the converted substrate (compare to Figure 2C) was extended partially or fully by Klenow. For BLM and Klenow-containing reaction mixtures with the Holliday junction substrate, the percentage of extension of the labeled strand was lower (~25%) than with WRN-E84A, consistent with a lower level of BLM-mediated conversion under these conditions (compare panels C and D of Figure 2).

We also performed similar reactions on our Holliday junction substrate with WRN-E84A and human polymerase δ (Figure 5C). As expected, no extension was observed with WRN-E84A alone (lane 2), but some short extension products are visible with polymerase δ alone (lane 3); these products probably result from removal of the unpaired nucleotides at the 3′ end of the labeled strand by the 3′ to 5′ proofreading function of polymerase δ followed by fill-in synthesis on the unconverted Holliday junction. Because of this possibility, the origins of similar short extension products in reaction mixtures containing both WRN-E84A and polymerase δ are unclear. Nevertheless, significant amounts of the 122−123-nucleotide full extension product are also observed in these reactions (Figure 5C, lane 4, denoted by the white arrow). As in reactions described above with Klenow, this product reflects synthesis beyond the replication fork junction to the end of the leading parental template strand, and its presence confirms that WRN-E84A mediates conversion of the Holliday junction to four-stranded replication fork and allows access to the 3′ end of the leading daughter strand and DNA synthesis by polymerase δ. In similar reactions on the Holliday junction substrate, these full extension products were also detected only in reaction mixtures containing both BLM and polymerase δ (Figure 5 of the Supporting Information) but were present at much lower levels than with WRN-E84A and polymerase δ. Importantly, these experiments confirm that WRN and BLM are capable of re-establishing a functional replication fork from a Holliday junction structure reflective of a regressed fork.

DISCUSSION

RecQ helicases are postulated to participate in pathways that respond to replication fork stalling caused by DNA damage or other circumstances. Accumulating evidence strongly suggests that the human RecQ helicases WRN and BLM (deficient in WS and BS, respectively) function with respect to DNA replication in a manner that maintains genomic stability. In the

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absence of WRN or BLM function, replication abnormalities are observed, including aberrant fork progression dynamics,[18,20,31] and the frequency of chromosomal aberrations is dramatically increased. WRN- and BLM-deficient cells have been shown to be hypersensitive to agents that block replication fork progression, including HU, aphidicolin, and interstrand cross-linkers.[23,24,28,30,31] In wild-type cells, WRN and BLM are recruited to replication foci in response to certain types of DNA-damaging treatments.[25−27,29,33] Taken together, these studies indicate that WRN and BLM function in response to replication fork blockage. Logically, cells would want to avoid the collapse of replication forks that would result in double-strand breaks and unscheduled recombination events. Indeed, blocked forks seem to be stabilized (in a manner dependent upon S phase checkpoint factor ATR) for a period of time after treatment with the replication inhibitor HU; in the absence of ATR function or after prolonged HU incubation, double-strand breaks eventually form, presumably because of replication fork collapse.[54−56] Although the DNA remodeling events that might occur immediately upon replication blockage in vivo remain unclear, some models propose regression of forks involving repairing of parental strands and annealing of nascent daughter strands to form a chicken foot or Holliday junction intermediate. If so, the seemingly least problematic scenario for replication restart would be for the regressed Holliday junction intermediate to be converted (by "reverse" branch migration) back to a fork structure competent for DNA synthesis. In this study, we have examined whether WRN and/or BLM might function in this process.

Biochemical experiments by several groups indicate that WRN and BLM are ATPases and helicases and prefer to bind to and act on complex three- and four-stranded DNA structures, including Holliday junctions.[39,40,46,57] Specifically, WRN and BLM bind to and unwind Holliday junctions of limited mobility and are able to perform branch migration over long distances on mobile Holliday junction structures.[25,38−40] Thus, their enzymatic activities, in combination with evidence regarding roles for these proteins in response to replication blockage, implicate WRN and BLM as possible candidate factors that might catalyze this reverse branch migration of "regressed" Holliday junctions to regenerate functional replication forks. Here, the actions of BLM and WRN proteins were examined on a special Holliday junction substrate capable of being converted to a four-stranded replication fork structure. In reaction mixtures containing ATP, WRN-wt, exonuclease-deficient WRN-E84A, and BLM independently convert this Holliday junction to a replication fork structure. Substitution of ATPγS or use of ATPase- and helicase-deficient BLM-D795A or WRN-K577M mutant protein in these reactions prevented conversion, demonstrating the requirement for ATP hydrolysis for this process. On this particular Holliday junction structure, conversion to the fork is the predominant reaction using either WRN-E84A or BLM; ATP-dependent unwinding or disruption of the Holliday junction to other products occurs at lower levels. Also, the conversion from Holliday junction to four-stranded fork by WRN-E84A and BLM was substantially influenced by the absolute concentration of $MgCl_2$, as was the binding of WRN-E84A to the Holliday junction substrate. Because increasing Mg^{2+} concentrations favor the stacked X conformation of Holliday junction structures over the square planar conformation,[43,44] our results indicate that WRN (and BLM) preferentially binds to and acts on the square planar (open) conformation of Holliday junctions. This is also consistent with data showing that spontaneous branch migration of mobile Holliday junctions occurs much faster when these junctions are in the open conformation as compared with the stacked X conformation.[58] Notably, the 3′ to 5′ exonuclease activity of WRN-K577M (and WRN-wt) was very evident in reactions performed with the Holliday junction substrate. Because WRN-K577M cannot unwind or branch migrate this substrate, its exonuclease activity is clearly targeted to the 3′ end of the leading daughter strand in the context of this Holliday junction reflective of a regressed replication fork. This result suggests that WRN exonuclease activity might be involved in processing the leading daughter strand following fork regression. Such an activity could be useful for removing unpaired nucleotides potentially misincorporated by replicative or translesion polymerases due to obstructions in the template that might be the triggers for fork regression.

This evidence indicates that, when presented with a Holliday junction substrate capable of being either unwound or branch migrated, WRN and BLM preferentially catalyze branch migration. Thus, it was relevant to determine the action of another established branch migration enzyme, human RAD54, on our Holliday junction substrate. Notably, a very recent paper from Mazin and colleagues, using an assay similar to ours, demonstrated that RAD54 catalyzes conversion of a Holliday junction to replication fork, as did BLM.[48] Similarly, we show here that RAD54 can convert our Holliday junction substrate to a four-stranded replication fork. However, compared to the low level of conversion we observe with RAD54, WRN and BLM appear to preferentially mediate conversion of our Holliday junction to the four-stranded replication fork species. WRN appears to initially target the Holliday junction structure, as addition of Holliday junction binding protein RuvA markedly inhibits the conversion reaction. We speculate that BLM may have an even higher affinity for Holliday junctions than WRN (and even RuvA), because its action is more weakly inhibited by RuvA and higher Mg concentrations. This is also in agreement with studies showing a specific role for BLM, along with TOP3, RMI1, and RMI2, in the processing of Holliday junction structures.[59,60] Our results add further support to the concept that, in vivo, ATP hydrolysis by WRN, BLM, and perhaps other RecQ family members is primarily utilized to drive branch migration instead of simple duplex unwinding. However, human RECQ4 was not able to catalyze the conversion of Holliday junctions to the replication fork, although it could mediate a complementary strand-dependent unwinding or strand exchange reaction. This may suggest the involvement of the RQC and/or HRDC domains (that RECQ4 lacks) in helping mediate this activity, and it certainly further separates the function of RECQ4 with those of WRN and BLM. Although our specific conversion assay seems to exhibit preferential activity by WRN-E84A and BLM, further research is needed to clarify physiological specificity in the putative re-establishment of replication forks from regressed Holliday junctions.

The physiological importance of this conversion process would be to re-establish a viable four-stranded replication fork suitable for resumption of normal bidirectional replication. Therefore, we examined whether the primary product from our WRN-E84A- and BLM-mediated conversion reactions was capable of being acted upon by DNA polymerases. To simplify analysis of the reaction products, we primarily used 3′ to 5′ exonuclease-deficient Klenow fragment in our experiments.

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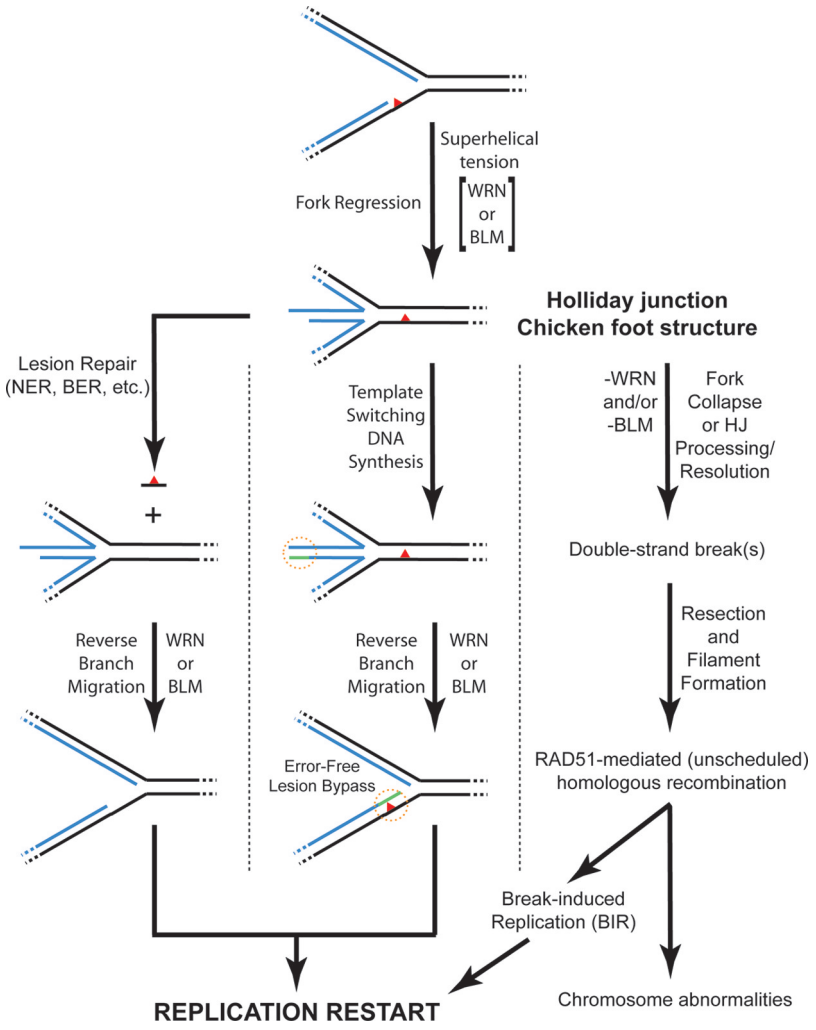

Figure 6. Model for action(s) of WRN and BLM during resolution of replication blockage. In the presence of certain lesions or obstructions (denoted by a red triangle) on the parental strands, replication fork progression is inhibited by blockage of replicative synthesis of the relevant daughter strand. The blocked fork may then be subject to regression to generate a Holliday junction or chicken foot structure, aided physically by superhelical tension (positive supercoiling) generated ahead of the fork during DNA replication and/or enzymatically by WRN or BLM. In the left pathway, the regressed fork regenerates a normal DNA duplex in the vicinity of the lesion, facilitating its recognition and removal by standard repair pathways, including nucleotide excision repair (NER) and base excision repair (BER). Reverse branch migration, catalyzed by WRN or BLM, re-establishes the replication fork structure, now without the blocking lesion. In the center pathway (regions of particular interest denoted with a dotted orange circle), extension of the unblocked daughter strand prior to regression provides for template switching and limited synthesis of the blocked daughter strand (colored green) once regression has occurred. In this case, reverse branch migration catalyzed by either WRN or BLM results in error-free lesion bypass (with the lesion presumably repaired at a later time). In these scenarios, WRN- or BLM-mediated re-establishment of the normal replication fork structure facilitates replication restart without replication errors or generation of double-strand breaks. In the right pathway, in the absence of WRN or BLM, replication forks collapse either directly or through processing or resolution of regressed Holliday junction structures, resulting in formation of double-strand breaks. These breaks serve as substrates for RAD51-mediated homologous recombination processes that can potentially restart replication through the break-induced replication (BIR) pathway. However, these unscheduled recombination events may be prone to errors, leading to the types of chromosomal aberrations associated with WRN or BLM deficiency. Throughout the diagram, parental and daughter strands are colored black and blue, respectively, with continuous regions of DNA indicated by dotted lines.

While the initial Holliday junction substrate was incapable of being acted upon by Klenow alone, incubation of WRN or BLM followed by Klenow (in the presence of ATP and dNTPs) resulted in extension of the labeled strand. The extent of extension (122−123 nucleotides, i.e., to the end of the leading parental strand template) in these reactions was identical to that observed when Klenow alone was incubated with control fork and partial duplex substrates. We also observed this full-length extension product in reaction mixtures containing the Holliday junction substrate, WRN-E84A, and the replicative T4 DNA polymerase (data not shown). Importantly, we could also readily observe this 122−123-nucleotide extension product in reactions performed on the Holliday junction with WRN-E84A and human DNA polymerase δ. In similar reactions performed with BLM and DNA polymerase δ on the Holliday junction substrate, this extension product was detectable but present at very low levels. Taken together, these results (1) confirmed that the product generated from the Holliday junction by WRN and BLM was indeed a replication fork structure and (2) demonstrated that the leading daughter strand of this structure could be fully extended by Klenow and by polymerase δ. It is not yet clear why better extension was observed with WRN-E84A than with BLM, considering that four-stranded forks would be expected to remain excellent

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substrates for both BLM and WRN binding.[46,57,61] However, we speculate that weaker extension of the converted replication fork by both Klenow and polymerase δ observed in the presence of BLM may be the result of steric hindrance. In agreement, direct extension of the leading daughter strand of four-stranded fork substrate by Klenow was increasingly inhibited by increasing BLM concentrations (data not shown). Also, a physical and functional interaction between WRN and polymerase δ, as previously reported,[62−64] may contribute to the higher level of extension observed in these reactions compared to those with BLM and polymerase δ. Regardless, our results show that DNA polymerases can act upon four-stranded replication forks re-established by either WRN or BLM from Holliday junctions reflective of fork regression.

Our results suggest that WRN or BLM might act to restore functional replication forks following replication fork blockage and regression to Holliday junction or chicken foot structures. It has been suggested that, subsequent to fork stalling in vivo, regression might be a consequence of positive supercoiling built up ahead of the fork.[65] However, previous studies in our lab and others suggest that WRN and/or BLM may assist with fork regression.[46,61,66] Thus, WRN or BLM may be involved in both fork regression and conversion of the regressed fork back to a functional replication fork, i.e., perhaps serving as an ATPase-driven swivel during resolution of replication blockage. In general agreement with this concept, in our experiments with both BLM and WRN, conversion of Holliday junction to four-stranded replication fork appears to plateau at <50% production of the fork product, suggesting that an equilibrium is reached in vitro between conversion to the fork product and regression back to the Holliday junction structure. This idea is also consistent with the results of Mazin and colleagues on BLM and RAD54[48] and our experiments showing that WRN binds with high and nearly equal affinity to Holliday junction and four-stranded fork structures, with a much lower affinity for partial duplexes and single-stranded DNA (A. Machwe and D. K. Orren, unpublished results).

On the basis of the existing evidence, we suggest the following model for participation of WRN or BLM in resolution of replication blockage (Figure 6). When replication forks are stalled by DNA lesions or other circumstances, fork regression to generate a Holliday junction occurs (possibly driven by superhelical tension with enzymatic assistance from WRN or BLM). Subsequently, the lesion or obstruction is removed from the reconstituted parental duplex (left pathway) or template switching occurs, in this case using the extended lagging daughter strand as a template for synthesis of the leading daughter strand (center pathway). Following these events, WRN or BLM catalyzes "reverse branch migration", i.e., the conversion of the Holliday junction back to a four-stranded replication fork upon which bidirectional DNA synthesis can be restarted. It should be reiterated here that all of these events are likely downstream of and controlled by the ATR-dependent S phase checkpoint pathway. In the absence of functional WRN or BLM (right pathway), replication blockage results in double-strand break generation, most likely through direct collapse of stalled forks and/or processing or resolution of Holliday junctions formed by fork regression. These double-strand breaks serve as substrates for RAD51-mediated recombination processes that, through the break-induced replication pathway, might restart replication. In agreement, cells deficient in WRN or BLM show increases in the number of (spontaneous or damage-induced) RAD51 foci,[23,67−69] and certain phenotypes of WRN-deficient cells are rescued by introduction of ectopic Holliday junction resolvase enzymes.[67,70] In most instances, the alternate RAD51-mediated pathway would probably be error-free because of the presence of completely homologous partners. However, it might be expected that such recombinational repair processes would be more error-prone if blockage occurred within sequence elements that are highly repeated within the genome. Intriguingly, BLM-deficient cells have a high degree of instability in their repetitive rDNA arrays,[71] while WRN- and BLM-deficient cells appear to have substantial telomeric instability.[72−74] Even though the phenotypes of WS and BS are distinct, some evidence (including the results presented here) hints at partially redundant roles for WRN and BLM in resolution of replication blockage. However, both WRN and BLM might also participate in other replication- or recombination-related transactions. With regard to BLM, it seems to have both anti- and pro-recombinogenic roles. Its pro-recombinogenic roles may be as a factor enhancing double-strand break resection[75−77] and/or as part of a complex (including TOP3α, RMI1, and RMI2) that acts late in a recombination pathway to suppress sister chromatid exchange.[59,60] Additional research is needed to specify and confirm the DNA metabolic steps at which these enzymes act in vivo.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Sequences of oligonucleotides used in construction of DNA substrates utilized in this study (Table 1), the action of the ATPase- and helicase-deficient WRN-K577M mutant on our specialized Holliday junction substrate (Figure 1), the effect of $MgCl_2$ concentration on WRN-E84A-mediated unwinding of a partial duplex (Figure 2), the effect of increasing $MgCl_2$ concentration on BLM- and RAD54-mediated conversion of Holliday junction to four-stranded replication fork (Figure 3), depiction of how human RPA has a minimal influence on the conversion of Holliday junction to replication fork by WRN-E84A (Figure 4), depiction of how human DNA polymerase δ can detectably catalyze extension of 4-stranded replication fork converted by BLM (Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ATPγS, adenosine 5′-*O*-(thio)triphosphate; BS, Bloom syndrome; EMSA, electrophoretic mobility shift assay; HU, hydroxyurea; Klenow, Klenow fragment of *E. coli* DNA polymerase I (for these studies, 3′ to 5′ exonuclease-deficient);

PAGE, polyacrylamide gel electrophoresis; RTS, Rothmund-Thomson syndrome; SE, standard error; WS, Werner syndrome.

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