FANCJ Uses Its Motor ATPase to Destabilize Protein-DNA Complexes, Unwind Triplexes, and Inhibit RAD51 Strand Exchange*

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Mutations in the FANCJ helicase predispose individuals to breast cancer and are genetically linked to the Fanconi anemia (FA) complementation group J. FA is a chromosomal instability disorder characterized by multiple congenital anomalies, progressive bone marrow failure, and high cancer risk. FANCJ has been proposed to function downstream of FANCD2 monoubiquitination, a critical event in the FA pathway. Evidence supports a role for FANCJ in a homologous recombination pathway of double strand break repair. In an effort to understand the molecular functions of FANCJ, we have investigated the ability of purified FANCJ recombinant protein to use its motor ATPase function for activities in addition to unwinding of conventional duplex DNA substrates. These efforts have led to the discovery that FANCJ ATP hydrolysis can be used to destabilize protein-DNA complexes and unwind triple helix alternate DNA structures. These novel catalytic functions of FANCJ may be important for its role in cellular DNA repair, recombination, or resolving DNA structural obstacles to replication. Consistent with this, we show that FANCJ can inhibit RAD51 strand exchange, an activity that is likely to be important for its role in controlling DNA repair through homologous recombination.

A growing interest in the mechanisms of helicases in cellular nucleic acid metabolism has been partly fueled by the knowledge that an increasing number of human diseases are genetically linked to mutations in genes encoding helicase-like proteins. RecQ helicases are prominent in this category, since three diseases of premature aging and/or cancer are attributed to recessive mutations in genes that encode bona fide DNA helicases (Werner and Bloom Syndromes) or DNA-dependent ATPase (Rothmund-Thomson Syndrome) (1, 2). Other helicase disorders exist, including Fanconi anemia (FA),\(^3\) which is a recessive genetic disorder characterized by multiple congenital anomalies, progressive bone marrow failure, and high risk of cancer (for a review, see Ref. 3). Among the 13 FA complementation groups from which all of the FA genes have been cloned, the FANCM and FANCJ genes encode DNA-stimulated ATPases, the latter also being a Superfamily 2 DNA helicase (4). The interaction of FANCJ with BRCA1 (5) and the existence of FANCJ mutations in early onset breast cancer patients (5, 6) as well as its genetic linkage to the FA-J complementation group (7–9) have clarified that FANCJ is a tumor suppressor.

Although FANCJ helicase activity has been studied biochemically, the cellular functions of FANCJ in DNA repair are likely to require additional functions that involve protein–protein and protein–DNA interactions. Two protein partners of FANCJ that appear to be important are the single-stranded DNA-binding protein RPA, which stimulates FANCJ helicase activity (10), and the mismatch repair complex MutL\(\alpha\), to which FANCJ binds and enables FANCJ to perform its DNA repair function (11). The FANCJ helicase functions downstream of FANCD2 monoubiquitination, a critical event in the FA pathway (9, 12). Evidence supports a role of FANCJ in a homologous recombination (HR) pathway of double strand break repair (5, 9). FA-J null cells are hypersensitive to DNA interstrand cross-linking (ICL) agents (9). This may reflect defective HR in the late steps of processing DNA ICLs. FANCJ helicase unwinds duplex DNA with a 5’ to 3’ directionality, preferentially binds and unwinds forked duplexes, and has the ability to unwind the invading strand of a three-stranded D-loop structure, a key early intermediate of HR repair (13, 14). Understanding how FANCJ performs its role(s) in DNA repair through its coordination of catalytic activity and protein interaction is an important topic of

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3 The abbreviations used are: FA, Fanconi anemia; HR, homologous recombination; ICL, interstrand cross-linking; ssDNA, single-stranded DNA; WT, wild type; PIPES, 1,4-piperazinediethanesulfonic acid; nt, nucleotide(s); scDNA, supercoiled DNA; ATPγS, adenosine 5’-O-(thiotriphosphate).

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Novel Biochemical Functions of FANCJ Motor ATPase

investigation, since this will provide a better comprehension of the FA pathway.

In an effort to better understand the molecular functions of FANCJ, we explored the hypothesis that FANCJ may use its motor ATPase for other catalytic activities in addition to DNA unwinding of canonical B-form duplex DNA. For example, nonconventional roles of FANCJ ATPase motor may be important for its role in replication initiation after DNA damage (15).

The recent demonstration that FANCJ helicase activity is required for timely progression through S phase (16) suggests that the helicase plays a role in resolving alternate DNA structures that impede the replication fork. Recently, we reported that FANCJ can efficiently resolve G-quadruplex DNA structures (17). The DOG-1 protein in nematodes, which has sequence similarity to the FANCJ helicase domain, has been proposed to have a role in the resolution of G4 DNA structures based on the observation that dog-1 (deletions of guanine-rich DNA) mutations show germ line as well as somatic deletions in genes containing guanine-rich DNA (18).

Another source of genomic instability and perturbation to cellular processes of nucleic acid metabolism is the DNA triple helix, an alternate DNA structure stabilized by Hoogsteen hydrogen bonding of the third strand to the DNA double helix that poses a source of genomic instability and perturbs cellular processes of nucleic acid metabolism (19, 20). For example, triplex formation by the Friedreich ataxia (GAA)n repeats results in reduced expression of the frataxin gene (21), inhibits DNA replication in vitro (21, 22), and causes replication forks to stall at (GAA)n repeats in vivo (23).

In addition to resolving alternate DNA structures, the ability of certain helicases to clear proteins from nucleic acid structures is potentially important, since helicases provide access of single-stranded DNA or RNA for subsequent steps during vital processes, such as replication, recombination, repair, transcription, and translation (24). A limited number of helicases have been characterized for their ability to catalyze protein displacement from DNA in an ATP-dependent manner. A classic example is the yeast Srs2 helicase, which prevents recombination by disrupting Rad51 nucleoprotein filaments (25, 26). Human BLM (27) and RECQL5 (28) DNA helicases have also been shown to strip human RAD51 from DNA. Similarly, Escherichia coli UvrD helicase dismantles RecA nucleoprotein filaments formed on ssDNA (29) and clears proteins bound to replication forks (30). Bacteriophage T4 Dda helicase has the ability to dissociate a stationary RNA polymerase (31) or E. coli trp repressor bound to DNA (32). The yeast Pif1 helicase acts as a negative regulator of telomerase by catalytically removing telomerase from telomeric DNA (33). Proteins with the canonical ATPase/helicase motifs, such as chromatin remodeling enzymes, can alter histone-DNA interactions (34). A common feature of chromatin remodeling enzymes and helicases is that both classes of proteins utilize the energy from ATP hydrolysis to perform their protein displacement function.

In this study, we have experimentally determined that FANCJ utilizes its motor ATPase function to resolve a DNA triple helix. We also demonstrate that FANCJ disrupts a model protein-DNA substrate interaction of streptavidin bound to a biotinylated ssDNA molecule. This approach has been successfully used to characterize a limited number of helicases or motor ATPases to perform protein-nucleic acid remodeling functions (for reviews, see Refs. 24 and 35). The identification and characterization of a novel FANCJ protein displacement activity suggests that the current model(s) proposed for FANCJ function in the DNA damage response will be enhanced with the new appreciation that FANCJ efficiently disrupts a model protein-DNA interaction of high affinity. In support of this model, we further demonstrate that FANCJ inhibits RAD51 strand exchange, a reaction that is likely to be important to prevent inappropriate homologous recombination.

MATERIALS AND METHODS

Proteins—Baculovirus encoding FANCJ wild type (WT) or FANCJ-M299I with a C-terminal FLAG tag was used to infect High Five insect cells, and the recombinant FANCJ protein was purified as previously described (13). Recombinant FANCJ protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. Purified recombinant FANCJ-WT and FANCJ-M299I proteins predominantly migrated as single bands of the predicted size (130 kDa) on Coomassie-stained SDS-polyacrylamide gels and judged to be greater than 90% pure (data not shown). At least three independent protein purifications of the purified wild-type and mutant recombinant FANCJ proteins were performed, yielding specific ATPase activities in the presence of the forked duplex DBA effector comparable with those previously published (36), 25 min⁻¹ for FANCJ-WT and 170 min⁻¹ for FANCJ-M299I. Recombinant WRN (37) and RECQ1 (38) proteins were purified as previously described. Human RAD51 and RPA proteins were purified as previously described (27).

DNA Substrate Preparations—PAGE-purified oligonucleotides used for the preparation of DNA substrates were purchased from either Lofstrand Laboratories (Gaithersburg, MD) or Midland Certified Reagent Co. (Midland, TX) and are listed in Table 1. Oligonucleotides were 5’-32P-end-labeled, and forked duplex or 5’-tailed DNA substrates were prepared as previously described (39).

For triplex DNA substrates with a pyrimidine third strand, the plasmid pSupF5, which contains a duplex sequence that serves as a target for a third strand designated TC30, was used (40). Cleavage of the plasmid with Ndel released fragments of 4 and 0.6 kilobase pairs. The triplex site lies 1800 bases from one end of the large fragment. Triplexes were prepared by incubation of 3 pmol of the indicated 5’-32P-labeled TC30 oligonucleotide or TC30 with a 15-nt 5’-ssDNA tail, designated 5’-15nt-TC30 (Table 1) (based on the molecular weights of the reaction components) overnight at room temperature with 6 pmol of Ndel-cleaved plasmid in triplex annealing buffer containing 33 mM Tris acetate (pH 5.5), 66 mM KAc, 100 mM NaCl, 10 mM MgCl₂, and 0.1 mM spermine. The complexes were then separated from unbound oligonucleotide by gel filtration chromatography using Bio-Gel A-5 M resin equilibrated in triplex annealing buffer. Triplex substrates were stored at 4°C for 1 week and found to be stable for functional assays.

Restriction protection of the XbaI site, which resides in the triplex target site was performed and demonstrated for the
restriction fragment-based triplex substrate, as previously described (40). Briefly, triplex complexes isolated by gel chromatography were separated from free oligonucleotide by ethanol precipitation from 2.5 M ammonium acetate. They were suspended in 50 mM NaCl, 10 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM spermine, 100 µg/ml bovine serum albumin and digested with XbaI at 37 °C.

The 5′-tailed blunt triplex substrate with a pyrimidine third strand, which contained a triplex region without underlying duplex extensions on either side of the triplex site, consisted of a 30-bp duplex (TC30W and TC30C; Table 1) annealed to 100 pmol of the purine third strand except 10 pmol of duplex was annealed to 100 pmol of the purine third strand (TC30PUR).

Triplex substrates were constructed containing a purine third strand with underlying duplex that had either a purine strand (5′-15nt-PUR30W) or a pyrimidine strand (5′-15nt-PYR30C). For both substrates, the purine strand of the underlying duplex was radiolabeled. The radiolabeled 5′-15nt-PUR30W or 5′-15nt-PYR30C was annealed to PRY30C or 5′-15nt-PYR30C, respectively, at equimolar concentrations. Annealing was performed under the same buffer conditions and temperature as the corresponding 5′-tailed triplex substrate with a purine strand, except 10 pmol of duplex was annealed to 100 pmol of the purine third strand (TC30PUR).

Streptavidin Displacement Assays—Streptavidin displacement reaction mixtures (20 µl) contained 40 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2% glycerol, 100 ng/µl bovine serum albumin, 2 mM ATP, 10 fmol of the specified DNA substrate (0.5 mM final substrate concentration), and the indicated concentrations of FANCJ helicase. For streptavidin displacement reactions, 10 fmol of DNA substrate was preincubated with 100 nM streptavidin (Sigma) for 10 min at 37 °C. Streptavidin bound to the DNA substrate was detected by a slower migrating gel-shifted species on nondenaturing 12% polyacrylamide gel. Reactions were initiated by adding FANCJ prior to the addition of biotin (1 µM) and incubated at 30 °C for 15 min (unless otherwise noted), followed by a quench with the addition of 10 µl of stop buffer (50 mM EDTA, 40% glycerol, 0.9% SDS, 0.05% bromphenol blue, and 0.05% xylene cyanol). Products were resolved on nondenaturing 12% (19:1 acrylamide/bisacrylamide) polyacrylamide gels and quantitated. A PhosphorImager was used for detection, and the ImageQuant software (GE Healthcare) was used for quantitation of the reaction products. The percentage displacement was calculated. The data represent the mean of at least three independent experiments, with S.D. values shown by error bars.

**Duplex DNA Helicase Assays**—RECQ1 and BLM helicase assays with forked duplex substrates were performed under RECQ1 reaction conditions, as described previously (38). WRN helicase assays were performed as described previously (39). FANCJ helicase assays were performed as described previously (14).

**Triplex Displacement Assays**—Reaction mixtures (20 µl) contained 40 mM Tris-HCl (pH 7.4), 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2% glycerol, 100 ng/µl bovine serum albumin, 10 fmol of the specified triplex DNA substrate (0.5 mM DNA substrate concentration), and the indicated FANCJ concentrations. Reactions were initiated by the addition of FANCJ and then incubated at 30 °C for 15 min. A 10-µl aliquot of loading buffer (40% glycerol, 0.9% SDS, 0.1% bromphenol blue, 0.1% xylene cyanol) was added to the mixture at the end of incubation, and products were resolved on nondenaturing polyacrylamide gels visualized with a PhosphorImager and quantitated using ImageQuant software (GE Healthcare).

**ssDNA Protection Assays**—To form the nucleoprotein filament, 32P-labeled ssDNA (number 1; 5′-ACA GCA CCA GAT TCA GCA ATT AAG CTC TAA GCC ATC CGC AAA AAT GAC CTC TTA CTA AAA GGA-3′; 3 µM, nucleotides) was...
incubated with human RAD51 protein (1 μM) in buffer containing 25 mM Tris acetate (pH 7.5), 2 mM ATP, 1 mM magnesium acetate, 2 mM DTT, bovine serum albumin (100 μg/ml), 20 mM phosphocreatine, and creatine phosphokinase (30 units/ml) for 15 min at 37 °C. Then FANCJ (in the indicated concentrations) was added to the reaction mixture (10 μl, total) followed by incubation for 10 min. Finally, 1 μl of E. coli exonuclease I (0.25 units; New England Biolabs) was added to the reaction mixture, followed by a 15-min incubation. The products were deproteinized, analyzed by electrophoresis in a 10% nondenaturing polyacrylamide gel, visualized, and quantified using a Storm 840 PhosphorImager (GE Healthcare).

Inhibition of D-loop Formation by FANCJ—The procedure for monitoring D-loop formation was as previously described (27). In brief, to form the nucleoprotein filament, 32P-labeled ssDNA (number 90; Table 1; 3 μM, nucleotides) was incubated with human RAD51 protein (1 μM) in buffer containing 25 mM Tris acetate (pH 7.5), 1 mM ATP, 1 mM magnesium acetate, 2 mM dithiothreitol, bovine serum albumin (100 μg/ml), 20 mM phosphocreatine, and creatine phosphokinase (30 units/ml) for 15 min at 37 °C. FANCJ (in the indicated concentrations) and RPA (200 nM) were added, followed by a 10-min incubation. Then 2 mM calcium chloride was added, and the reactions were incubated for another 10 min. D-loop formation was initiated by the addition of pUC19 supercoiled DNA (scDNA) (50 μM, nucleotides). D-loop formation was terminated after a 15-min incubation. The products were deproteinized in the presence of 1.5% SDS and proteinase K (800 μg/ml) for 15 min at 37 °C and then mixed with 0.10 volume of loading buffer (70% glycerol, 0.1% bromphenol blue), analyzed by electrophoresis in 1% agarose gels, visualized, and quantified using a Storm 840 PhosphorImager (GE Healthcare).

RESULTS

FANCJ Displaces Streptavidin Bound to an Internal Biotinylated Deoxyoligonucleotide in a Manner That Is Dependent on the Location of Conjugated Biotin—To investigate the possibility that FANCJ utilizes its motor ATPase function for catalytic activities other than DNA unwinding, we tested the ability of FANCJ to disrupt a model protein-DNA interaction of high affinity presented by a biotinylated DNA substrate prebound with streptavidin at a specific location. The streptavidin-biotin complex is an extremely strong interaction (Kd ~10−15 M) (41), and the diameter of a streptavidin tetramer, ~45 Å (42), has previously been shown to block double-stranded DNA unwinding by T7 Gene 4 (43), DnaB (44), and WRN (39) helicases when it is positioned on the single strand on which the enzyme translocates.

Increasing concentrations of FANCJ-WT were incubated in the presence of ATP with a radiolabeled oligonucleotide that had streptavidin bound to the biotin conjugated either 28 or 52 nt from the 5′-end of the 66-mer oligonucleotide. As shown in Fig. 1A, FANCJ-WT displaced streptavidin bound to the DNA substrate with biotin positioned 28 nt away from the 5′-end in a protein concentration-dependent manner. Approximately 60% displacement was achieved by 9.6 nM FANCJ-WT during the 15-min reaction incubation (Fig. 1C). When FANCJ was tested for streptavidin displacement from a similar substrate, except that the biotin was positioned 52 nt from the 5′-end, significantly greater percentages of displacement were observed at all FANCJ concentrations compared with the substrate with biotin located 28 nt from the 5′-end (Fig. 1, B and C). Quantitative analyses demonstrated that ~2-fold greater displacement was observed at a FANCJ concentration of 2.4 nM protein. These results demonstrate that FANCJ more efficiently displaces streptavidin from a biotinylated oligonucleotide...
that has a longer (by 24 nt) 5'-ssDNA segment from the 5'-end, a result that is consistent with its increased 5' to 3' helicase activity on partially duplex DNA substrates with a longer 5'-ssDNA tail on the partial duplex substrate (14).

**Streptavidin Displacement by FANCJ Is Dependent on ATP Hydrolysis**—The nucleotide requirement for FANCJ streptavidin displacement activity was evaluated (Fig. 2). In the absence of ATP or in the presence of either ADP or the poorly hydrolyzable ATP analogue ATPγS, FANCJ-WT failed to catalyze streptavidin displacement on DNA substrates with biotin positioned either 28 nt (Fig. 2A) or 52 nt (Fig. 2B) away from the 5'-end. In contrast, in the presence of ATP, FANCJ-WT (9.6 nM) catalyzed 60 and 85% displacement on the substrates with biotin positioned 28 or 52 nt from the 5'-end, respectively. These results indicate that streptavidin displacement by FANCJ is indeed ATP-dependent. We also observed that a catalytically inactive FANCJ-K52R ATPase domain mutant that is deficient in helicase activity (13) failed to displace streptavidin from either biotinylated substrate (data not shown), indicating that the function is dependent on intrinsic ATP hydrolysis catalyzed by FANCJ.

Kinetic analysis demonstrated that streptavidin displacement activity by FANCJ was dependent on the time of incubation throughout a 10-min time course (Fig. 2C). Under steady state conditions, the rate of streptavidin displacement activity by FANCJ-WT was ~2-fold greater for the substrate with biotin positioned 52 nt from the 5'-end compared with 28 nt from the 5'-end. These results are consistent with the results of experiments in which varying concentrations of FANCJ-WT were incubated with the streptavidin-bound biotinylated DNA substrates for a fixed incubation time of 15 min (Fig. 1).

**Streptavidin Displacement Is Not a General Function of Superfamily 2 Helicases**—To determine if other Superfamily 2 DNA helicases have
the ability to disrupt the high affinity interaction of streptavidin bound to biotinylated ssDNA, we tested human DNA helicases of the RecQ helicase family (RECQ1, BLM, and WRN). Both RECQ1 and BLM failed to displace streptavidin from the biotinylated oligonucleotide under conditions in which the enzymes unwound a 31-bp forked duplex substrate (Fig. 3, A and B). WRN was also unable to perform streptavidin displacement under conditions in which WRN digested and unwound the 31-bp forked duplex or unwound a shorter 19-bp forked duplex (Fig. 3C). The inability of WRN to displace streptavidin bound to biotinylated ssDNA is consistent with our earlier observation that streptavidin bound to the 3'-ssDNA overhang adjacent to a 19-bp duplex prevented WRN from unwinding the partial duplex substrate (39). These results demonstrate that FANCJ is able to catalyze streptavidin displacement, whereas the human RecQ helicases BLM, WRN, and RECQ1 fail to perform this activity under conditions that the RecQ helicases are able to unwind duplex DNA.

**FANCJ Displaces Streptavidin from a 5'-Tailed DNA Duplex That It Unwinds**—The ability of FANCJ to displace streptavidin from ssDNA raised the question of whether the helicase might use its motor ATPase function to displace protein bound to the double-stranded region of a DNA molecule that FANCJ unwinds. To address this question, we examined streptavidin displacement by FANCJ from a partial duplex DNA substrate with a 5'-ssDNA tail in which streptavidin was bound to the terminal nucleotide of the radiolabeled top strand at the 3'-blunt end. FANCJ was able to displace streptavidin from this 5'-tailed duplex DNA substrate in a reaction that was dependent on hydrolyzable ATP (Fig. 4). Under these conditions, FANCJ was also able to unwind the streptavidin-free 5'-tailed duplex substrate (data not shown). FANCJ displacement of streptavidin from the biotinylated 5'-tailed duplex was slightly less efficient than streptavidin displacement from biotinylated ssDNA (Fig. 4). FANCJ was not able to displace streptavidin from an entirely duplex DNA substrate that lacks the 5'-ssDNA tail required to initiate translocation (data not shown), consistent with previously published findings that FANCJ fails to unwind a blunt duplex DNA substrate (14). These results suggest that FANCJ can displace protein bound to
duplex DNA when a 5’-ssDNA tail is present on the substrate for FANCJ to initiate translocation.

**FANCJ-M299I Breast Cancer Variant Has Increased Streptavidin Displacement Activity**—To evaluate the biochemical effect of a helicase domain FANCJ missense polymorphism (M299I) associated with breast cancer (5) on enzymatic function, we analyzed its streptavidin displacement activity under identical reaction conditions and the same protein concentrations used for the characterization of FANCJ-WT. As shown in Fig. 5, FANCJ-M299I catalyzed streptavidin displacement from the DNA substrates with biotin positioned either 28 nt (A) or 52 nt (B) from the 5’-end in a protein concentration-dependent manner. As predicted

![Image of FANCJ displaces streptavidin from a 5'-single-stranded DNA tailed duplex.](image)

**FIGURE 4.** FANCJ displaces streptavidin from a 5’-single-stranded DNA tailed duplex. FANCJ-WT (19.2 nM) was incubated with 2 mM ATP or ATPγS as specified, and the indicated 5’-single-stranded tailed duplex (TSTEM + 15BIOT3 annealed to TSTEMCOMP) or single-stranded DNA substrate (TSTEM + 15BIOT3) (0.5 nM) that had streptavidin bound to biotin conjugate residing at the 3’-end of the radiolabeled strand. Reaction mixtures were incubated for 15 min as described under “Materials and Methods,” and products were resolved on native 12% polyacrylamide gels. The star indicates the position of the 5’-32P label. A representative gel from a typical experiment is shown. *Filled triangle*, heat-denatured DNA substrate control.

![Image of FANCJ-M299I mutation associated with breast cancer increases the ability of FANCJ to displace streptavidin from the biotinylated DNA substrate.](image)

**FIGURE 5.** M299I FANCJ mutation associated with breast cancer increases the ability of FANCJ to displace streptavidin from the biotinylated DNA substrate. The indicated concentration of FANCJ-M299I was incubated with 2 mM ATP and DNA substrate (0.5 nM) that had streptavidin bound to the covalently linked biotin moiety either 28 nt (A) or 52 nt (B) from the 5’-end of the radiolabeled oligonucleotide. Reaction mixtures were incubated for 15 min as described under “Materials and Methods,” and products were resolved on native 12% polyacrylamide gels. The star indicates the position of the 5’-32P label. Quantitative analyses of FANCJ-M299I streptavidin displacement with DNA substrate that had streptavidin bound to biotin conjugate residing either 28 nt (filled circles) or 52 nt (open circles) from the 5’-end are shown in C. For comparison, results from experiments with FANCJ-WT (Fig. 1C) are shown in the inset. D, kinetics of streptavidin tetramer displacement by 2.4 nM FANCJ-M299I in the presence of 2 mM ATP and DNA substrate (0.5 nM) that had streptavidin bound to the covalently linked biotin moiety either 28 nt (filled circles) or 52 nt (open circles) from the 5’-end of the radiolabeled oligonucleotide. Data represent the mean of at least three independent experiments with S.D. values indicated by error bars.
from the experiments with FANCJ-WT, the M299I variant acted more efficiently on the substrate with biotin located farther from the 5'\/-H11032-end (C). Moreover, on either substrate, FANCJ-M299I was significantly more active compared with FANCJ-WT at all protein concentrations that were below the plateau attributed to substrate depletion effects (Fig. 5, compare C with inset). A kinetic analysis of streptavidin displacement by FANCJ-M299I (2.4 nM) (Fig. 5D) and FANCJ-WT (9.6 nM) (Fig. 2C) demonstrated that FANCJ-M299I catalyzed streptavidin displacement with an 8-fold greater specific activity compared with FANCJ-WT on the substrate with streptavidin bound to the biotin 28 nt from the 5'//-H11032-end and a 5-fold greater activity on the substrate with streptavidin bound 52 nt from the 5’-end. These results demonstrate that the M299I mutation exerts a gain-of-function effect on ATP-dependent streptavidin displacement activity, a result that is consistent with the elevated $k_{cat}$ for ATP hydrolysis of the M299I variant as well as its significantly increased helicase activity on duplex DNA substrates with backbone discontinuity obstacles that impede FANCJ-WT (36).

**FANCJ Requires a 5’-ssDNA Tail on the Third Strand to Unwind Triplex DNA**—Although the DNA substrate specificity of FANCJ as a helicase has been characterized, little is known about its DNA translocation activity. The results from the streptavidin displacement assays using either biotinylated ssDNA or blunt duplex DNA suggested that FANCJ may require at least an ssDNA tail to load and translocate along a DNA molecule. To further address if FANCJ translocates along duplex DNA, we utilized the triplex displacement assay. The triplex DNA substrate consists of a pyrimidine motif third DNA strand (TC30), residing in the major groove of the DNA double helix, that is stabilized by Hoogsteen hydrogen bonding with the Watson-Crick base pairs.

First, we asked if FANCJ can disrupt a triplex substrate in which the 30-mer third strand was completely annealed to a target site within a 4-kb duplex restriction fragment (Fig. 6A). This “flush” substrate lacked a 3’- or 5’-ssDNA overhang on the third strand. FANCJ was inactive on this substrate, whereas the yeast RAD54 protein, a member of the Swi2/Snf2-like family of DNA-dependent ATPases and remodeling enzymes, was able to release the third strand (Fig. 6A), consistent with previously published data on RAD54 translocase activity (45). Thus, FANCJ does not behave as a classic double-stranded DNA translocase, such as RAD54 (45, 46) or FANCM (47).

We next asked if FANCJ might be active on a triplex substrate related to the flush substrate except that an additional 15-nt 5’-ssDNA tail was present on the third strand that might
FANCJ unwinds a DNA triple helix with a purine-rich third strand. A, the indicated concentration of FANCJ-WT was incubated with 2 mM ATP (lanes 1–8) or ATPγS (lane 9) and 0.5 mM 5′-tailed blunt triplex with a purine third strand for 15 min as described under “Materials and Methods,” and products were resolved on native 10% polyacrylamide gels. The star indicates the position of the 5′-32P label. B and C, FANCJ (38.4 nM) was incubated for 15 min in the presence of ATP or ATPγS with triplex substrates that contained a purine third strand with underlying duplex that had either a pyrimidine strand with a 15-nt 5′-tail (B) or a purine strand with a 15-nt 5′-tail (C). Representative gels from typical experiments are shown. Filled triangle, heat-denatured DNA substrate control. Duplex (lane 5 in B and C) is the radiolabeled underlying duplex of the triplex substrates.

enable FANCJ to load, since the enzyme was shown to be a 5′ to 3′ helicase on standard duplex DNA substrates. In this case, FANCJ was active on the 5′-tailed triplex substrate, and the third strand was released in a protein concentration-dependent manner (Fig. 6, B and D). Approximately 35% of the triplex was unwound at the highest FANCJ concentration tested, 19.2 nM. A 3′-tailed triplex substrate was not unwound (data not shown), indicating that FANCJ required a 5′-ssDNA tail on the triplex-forming oligonucleotide to unwind the third strand from the 4-kb duplex fragment. FANCJ helicase activity on the 5′-tailed triplex substrate was dependent on ATP hydrolysis, since no activity was detected in the presence of the poorly hydrolyzable ATP analog ATPγS (Fig. 6B). These results demonstrated that FANCJ unwinds the triplex-forming oligonucleotide from the 4-kb duplex fragment in an ATP-dependent reaction only when the third strand contains a 5′-ssDNA tail for FANCJ helicase to load on.

To assess the DNA structural elements important for FANCJ activity on the triplex DNA substrate, we tested FANCJ on a DNA triplex composed of a 30-bp duplex annealed to a pyrimidine-rich third strand flanked by a 15-nt 5′-ssDNA. Unlike the previous triplex substrate characterized by a 30-mer third strand annealed to a 4-kb duplex fragment, no underlying duplex extensions exist in the 5′-tailed blunt triplex substrate. As shown in Fig. 6C, FANCJ was able to unwind the 5′-tailed blunt triplex substrate in a protein concentration-dependent manner. No unwinding of the 5′-tailed blunt triplex substrate was detected in the presence of ATPγS (Fig. 6C). FANCJ helicase activity on the 5′-tailed blunt triplex substrate was comparable with that of the 5′-tailed triplex substrate with an extended underlying duplex at FANCJ concentrations up to 5 nM; however, more of the 5′-tailed triplex with an extended underlying duplex was unwound at 19 nM compared with the 5′-tailed blunt triplex (Fig. 6D). No unwinding of the blunt triplex substrate by FANCJ was detected when the third strand contained only a 3′-ssDNA tail or lacked both 3′- and 5′-ssDNA tails (data not shown). These experiments show that FANCJ can unwind the triplex without a requirement for a single-strand/double-strand fork at the junction of the third strand of the triplex substrate; however, a 5′-ssDNA tail on the triplex-forming oligonucleotide is required for FANCJ to unwind the third strand of the triplex structure.

FANCJ Unwinds a Triplex with a Purine-rich Third Strand—The class of triplexes used in Fig. 6 contain a pyrimidine-rich third strand annealed to a duplex with Watson and Crick strands that are also both pyrimidine-rich. However, this type of triplex cannot be formed at neutral pH unless cytosines of the third strand are modified (e.g. methylated). In contrast, triplexes can be formed at neutral pH in the presence of bivalent metals from an incoming purine-rich third strand that is annealed to a duplex that consists of a pyrimidine-rich strand and a purine-rich strand. This type of triplex may be more relevant to physiological conditions. Therefore, we constructed such a triplex with a purine-rich third strand and tested it for unwinding by FANCJ. FANCJ unwound the purine-rich triplex in a protein concentration-and hydrolyzable ATP-dependent manner (Fig. 7A).

The ability of FANCJ to release the third purine strand of the triplex substrate raised the question of whether the triplex oligonucleotide might interfere with FANCJ unwinding the Watson-Crick base pairs of the duplex target site. To address this possibility, we constructed triple helix DNA substrates in which the third invading purine strand was annealed to duplex that had either a pyrimidine strand with a 15-nt 5′-tail (Fig. 7B) or a purine strand with a 15-nt 5′-tail (Fig. 7C). The results from these experiments demonstrated that FANCJ can unwind the underlying duplex of the triplex much more efficiently when the helicase loads on the 5′-ssDNA tail of the purine-rich strand compared with loading on the 5′-ssDNA tail of the pyrimidine-rich strand.

FANCJ Destabilizes the RAD51 Nucleoprotein Filament—The ability of FANCJ to efficiently disrupt the high affinity interaction of streptavidin bound to biotinylated ssDNA suggested that FANCJ may use its ATPase-dependent function to destabilize the RAD51-ssDNA filament, which is an active species in recombinational DNA repair. To investigate this, we
employed a nuclease protection assay (Fig. 8A). Exonuclease I digestion of the ssDNA coated by RAD51 is protected from exonuclease I digestion (Fig. 8A). An increasing amount of FANCJ in the reaction (Fig. 8, lanes 4–9) digested by exonuclease I (lanes 1–3). These data are consistent with the ability of FANCJ to destabilize the RAD51-D loop formation showed dependence on the FANCJ concentration, with a half-inhibition (IC50) of ~70 nM of FANCJ (Fig. 9C). Thus, the data presented here suggest that the observed inhibition of D-loop formation is caused by destabilization of the RAD51-ssDNA filament by FANCJ.

**DISCUSSION**

In this study, we have identified and characterized two novel biochemical activities of FANCJ whereby the helicase utilizes its motor ATPase/translocase function to disrupt protein-DNA interactions and unwind alternate DNA triplex structures. FANCJ was able to efficiently disrupt a high affinity interaction of streptavidin bound to a biotinylated DNA substrate in a protein-concentration-, ATP-, and time-dependent manner. The efficiency of streptavidin displacement was found to be dependent on the location of the biotin moiety in the ssDNA molecule to which it is bound, indicating that a longer 5'-ssDNA loading dock facilitates optimal FANCJ displacement of the streptavidin moiety. FANCJ failed to displace streptavidin from a biotinylated blunt duplex DNA molecule, suggesting that a ssDNA region is necessary for the enzyme to productively load and translocate along DNA and displace the bound streptavidin. Consistent with this, we show that FANCJ unwind a triplex DNA substrate in an ATP-dependent reaction only when the triplex-forming oligonucleotide contained a 5'-ssDNA tail for FANCJ to load upon. Thus, FANCJ has the ability to resolve alternate DNA structures, such as triplexes (this study) or G-quadruplexes (17), that might impede the progression of the replication fork during S phase.

The requirement for a 5'-ssDNA tail, which FANCJ uses to load in order to translocate along DNA, is consistent with our earlier observations that FANCJ requires a pre-existing
D-loop formation was initiated by the addition of 2 mM CaCl₂ and pUC19 ssDNA to multiple FANCJ molecules binding to the ssDNA, which tract adjacent to the bound streptavidin. The effect may be due FANCJ is dependent on the length of the adjacent 5'-ssDNA that the apparent efficiency of streptavidin displacement by FANCJ is dependent on the length of the adjacent 5'-ssDNA tail distinguishes the catalytic activity of FANCJ from that of FANCM, another SF2 helicase-like protein, that displays classic triplex substrate only if the third strand contains a 5'-ssDNA tail (2).

The ability of FANCJ to displace the third strand of a DNA triplex substrate only if the third strand contains a 5'-ssDNA tail distinguishes the catalytic activity of FANCJ from that of FANCM, another SF2 helicase-like protein, that displays classic translocase activity on a flush triplex substrate (47).

There are several potential explanations for our observation that the apparent efficiency of streptavidin displacement by FANCJ is dependent on the length of the adjacent 5'-ssDNA tract adjacent to the bound streptavidin. The effect may be due to multiple FANCJ molecules binding to the ssDNA, which increase force production to drive streptavidin displacement, as is the case for Dda helicase, which functions by a cooperative inchworm mechanism (49). It is also possible that FANCJ assembly state or DNA binding is influenced by the ssDNA length. Further kinetic and biophysical studies will be required to better understand the requirements and mechanistic aspects of FANCJ translocase and unwinding activities.

One aspect of this study was to characterize the ability of a FANCJ polymorphic variant M299I in the conserved iron-sulfur cluster domain of FANCJ for its ability to disrupt the interaction of streptavidin with the biotinylated DNA substrate. This effort was instigated because several recent biological and biochemical observations suggest that the iron-sulfur cluster of FANCJ is a key regulatory domain of the protein. The FANCJ-A349P mutation resides immediately adjacent to the last cysteine of the iron-sulfur cluster and was identified in a patient with severe clinical symptoms of FA (8). The M299I substitution is adjacent to a conserved cysteine residue of the iron-sulfur cluster and represents a FANCJ polymorphic variant originally identified in cohort of women with early onset breast cancer and normal genotypes for BRCA1 and BRCA2 (5). Biochemical characterization of the purified recombinant FANCJ-M299I helicase revealed a significantly greater DNA-dependent ATPase activity compared with FANCJ-WT; moreover, the increased motor ATPase function of the M299I variant dramatically improved its ability to unwind DNA substrates with backbone discontinuity in the duplex region (36). The improved ability of FANCJ-M299I to displace streptavidin from the biotinylated DNA substrate suggests that the mutation might impact the function of FANCJ to remove proteins from DNA as well as unwind damaged DNA substrates.

How might the protein displacement function of FANCJ be important in a biological context? Monoubiquitination of FANC2 and FANCJ by the FA core complex in response to DNA damage or during S phase progression leads to the subsequent relocalization of FANCJ/FANC2 to chromatin (4). FANCJ and FANC2 are proposed to associate in DNA repair centers with other downstream proteins, including FANCD1/BRCA2, FANCN/PALB2, and FANCJ to regulate HR repair. FANCJ was found to form foci that co-localize with RPA in response to DNA damage or replicational stress (10) and show increased association with chromatin during S phase (16). FANCJ may utilize its motor ATPase function to scan for DNA damage, clear proteins bound to DNA repair/replication structures, disrupt recombination intermediates, or resolve alternate DNA structures that prevent replication fork progression.

Since RAD51 foci form in FA-J cells (9), it is suspected that FANCJ does not operate upstream of Rad51 foci formation during homologous recombinational repair. Rather, FANCJ may disrupt Rad51 nucleoprotein filaments in order to complete homologous recombinational repair or prevent untimely or promiscuous recombination between homologous sequences. By analogy, the yeast Srs2 helicase is believed to perform its function in DNA repair by stripping Rad51 from DNA (25, 26). More recently, the human BLM (27) and RECQL5 (28) helicases were also reported to strip RAD51 from DNA; however, the biological significance of precisely how this activity controls genetic recombination remains to be understood. In the cur-
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tient study, we have shown that FANCJ possesses the ability to destabilize the RAD51 nucleoprotein filament and inhibit the RAD51 strand exchange reaction, a biochemical activity that is likely to be important for its role in homologous recombinational repair. To our knowledge, this is the first demonstration that a 5′ to 3′ helicase performs this function, since previously, the SRS2, BLM, and RECQ5 helicases have all translocated 3′ to 5′. The WRN and RECQ1 helicase are not competent to inhibit RAD51 strand exchange (27, 28), indicating that this is not a generic function that all DNA helicases can perform.

Cells from FA-J patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA ICL agents; however, the precise role of FANCJ in the FA pathway remains to be elucidated. Recently, it was reported that FANCJ ATPase/helicase activity and binding to the mismatch repair complex MutLα are required to correct the FA-J cells’ ICL-induced 4 N DNA accumulation and sensitivity to ICLs (11). Conceivably, for ICL repair, mismatch repair complexes, including MutLα, mobilize or regulate FANCJ helicase activity to unwind DNA in the vicinity of the DNA damage to facilitate repair processes. FANCJ may also serve to displace a mismatch repair complex from DNA. In FA-J cells, the mismatch repair complex would be stuck or take longer to be displaced from DNA, leading to a prolonged G2/M arrest and/or delay in the completion of repair (11).

In yet another capacity for FANCJ to operate downstream in the FA pathway, it was proposed that FANCJ might promote translesion bypass at a stalled replication fork by removing proteins and creating a loading zone for the translesion polymerase (4). However, solid evidence for the involvement of FANCJ in any of these models is lacking. Nonetheless, the strong DNA damage-induced co-localization of FANCJ with the single-stranded DNA-binding protein RPA (10) and the enhanced association of the FANCJ complex with chromatin as cells progress through the S phase (16) provide further evidence that the enzyme functions in vivo on chromatized DNA substrates. FANCJ is likely to perform its catalytic functions in DNA repair and stabilization of the replication fork by displacing DNA-bound proteins or unwinding alternate DNA structures that interfere with normal DNA transactions and lead to genomic instability.

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