Interaction between the helicases genetically linked to Fanconi anemia group J and Bloom's syndrome

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Bloom's syndrome (BS) and Fanconi anemia (FA) are autosomal recessive disorders characterized by cancer and chromosomal instability. BS and FA group J arise from mutations in the *BLM* and *FANCF* genes, respectively, which encode DNA helicases. In this work, *FANCF* and *BLM* were found to interact physically and functionally in human cells and co-localize to nuclear foci in response to replication stress. The cellular level of *BLM* is strongly dependent upon *FANCF*, and *BLM* is degraded by a proteasome-mediated pathway when *FANCF* is depleted. *FANCF*-deficient cells display increased sister chromatid exchange and sensitivity to replication stress. Expression of a *FANCF* C-terminal fragment that interacts with *BLM* exerted a dominant negative effect on hydroxyurea resistance by interfering with the *FANCF*–*BLM* interaction. *FANCF* and *BLM* synergistically unwound a DNA duplex substrate with sugar phosphate backbone discontinuity, but not an 'undamaged' duplex. Collectively, the results suggest that *FANCF* catalytic activity and its effect on *BLM* protein stability contribute to preservation of genomic stability and a normal response to replication stress.

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Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by multiple congenital anomalies, progressive bone marrow failure, and high cancer risk (Levitus *et al*, 2006; Taniguchi and D'Andrea, 2006; Wang, 2007). Cells from FA patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA interstrand cross-linking agents. Thirteen causative genes (*FANCA/B/C/D1/D2/E/F/G/I/J/L/M/N*) have been identified to date. Increasing evidence suggests that these genes encode proteins that function as signal transducers (Andreassen *et al*, 2004; Pichierri and Rosselli, 2004; Matsuoka *et al*, 2007; Wang *et al*, 2007) and/or DNA processing molecules (Cantor *et al*, 2001; Meetei *et al*, 2005; Gari *et al*, 2008) in a DNA-damage response pathway. The FA proteins interact with a variety of DNA repair factors, and it is becoming increasingly evident that these interactions are critical for a robust DNA-damage response. For example, the Bloom's syndrome (BS) helicase (*BLM*) is associated with the FA core complex (Meetei *et al*, 2003) and *BLM* mutations lead to a disease characterized by severe growth retardation, immunodeficiency, reduced fertility, and predisposition to cancer (Hanada and Hickson, 2007). In recent work, *FANCM* was shown to act as a bridging protein to connect the *BLM* complex and FA core complex through independent domains that mediate physical interactions with *RM11/TopoIII α* and *FANCF*, respectively (Deans and West, 2009). At the cellular level, the hallmark of BS is a high rate of sister chromatid exchange (SCE) (Chaganti *et al*, 1974; Ray and German, 1984). An emerging theme is that *BLM* and its associated factors collaborate with FA proteins to deal with replication stress and maintain chromosomal stability. Moreover, recent evidence suggests that the FA pathway and *BLM* collaborate during mitosis to prevent micronucleation and chromosome abnormalities such as those at fragile sites where sister chromatid bridging can occur (Chan *et al*, 2009; Naim and Rosselli, 2009).

One of the more recently identified FA proteins, shown to be defective in the FA complementation group J, is the *BRCA1*-associated C-terminal helicase (*BACH1/BRIP*; designated here as *FANCF*) (Levitus *et al*, 2005; Levan *et al*, 2005; Litman *et al*, 2005). *FANCF* was also identified as a protein associated with breast cancer (Cantor *et al*, 2001; Seal *et al*, 2006). *FANCF*-deficient cells are sensitive to DNA cross-linking agents (Litman *et al*, 2005; Peng *et al*, 2007), and *FANCF*-depleted cells exhibit mitomycin C (MMC)-induced chromosomal breakage accompanied by triradial and quadriradial chromosome formation (Bridge *et al*, 2005; Litman *et al*, 2005). Like the human counterpart, chicken *brip1* (*FANCF*) cells are also characterized by chromosomal instability, including an elevated level of SCE (Bridge *et al*, 2005) that is not seen in most human FA cells. *FANCF* has been proposed to function downstream of *FANCD2* monoubiquitination (Litman *et al*, 2005), a critical event in the FA pathway.

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Evidence supports a role for FANCF in a homologous recombination (HR) pathway of double-strand break repair. However, FANCF is also required for timely progression through S phase (Kumaraswamy and Shiekhataar, 2007), and can unwind G-quadruplex structures to maintain genomic stability (London *et al*, 2008; Wu *et al*, 2008), suggesting that FANCF has additional roles in response to replication stress that may operate independently of the classic FA pathway (Wu *et al*, 2009; Hiom, 2010).

Since there is only limited information on the role of FA proteins in the proper DNA-damage response, investigation of protein interactions between endogenous FANCF and other DNA repair factors is likely to lead to new insights into the molecular pathogenesis of FA and the role of the FANCF helicase in reacting to replication stress and participating in HR repair. Given the evidence that both BLM and FANCF operate to maintain genomic stability in downstream events of HR repair, we investigated the potential interaction of the two helicases. Our studies demonstrate that FANCF and BLM are associated with each other *in vivo*, and that the two helicases physically and functionally interact with each other. These results are discussed in light of the cross-talk between the FA pathway and the BLM protein complex to prevent chromosomal instability associated with cancer and human disease.

Results

Reciprocal co-immunoprecipitation of FANCF and BLM helicases from human nuclear extracts

To explore whether endogenous FANCF and BLM are associated with each other *in vivo*, immunoprecipitation experiments were performed using HeLa cell nuclear extracts (NEs). Anti-FANCF antibody precipitated both FANCF and BLM from the NE (Figure 1A, lane 3). Neither FANCF nor BLM was precipitated when anti-FANCF antibody was omitted or when normal rabbit IgG was used (Figure 1A, lanes 4 and 5). In control experiments, the FANCF antibody immunoprecipitated BRCA1, a known protein partner of FANCF. However, the FANCF antibody failed to precipitate the Werner syndrome protein (WRN) (Figure 1A, lane 3), a RecQ helicase related to BLM in the conserved ATPase/helicase core domain (Sharma *et al*, 2006). FANCF and BLM were co-immunoprecipitated from NEs using an antibody directed against BLM protein (Figure 1A, lower panel).

As an additional control to address the specificity of the anti-BLM antibody, we tested for the co-precipitation of BLM and FANCF in isogenic BLM null and BLM corrected cell lines. Representative results from these experiments are shown in Figure 1B. Using the same antibody directed against BLM that was used for co-immunoprecipitation experiments with HeLa cell NEs, FANCF failed to be immunoprecipitated by the BLM antibody from BLM null cells, which lacked detectable BLM protein. In the control experiments, BLM and FANCF were co-immunoprecipitated from the BLM corrected cells.

We attempted similar experiments with NEs from FANCF null and corrected cells and a FANCF antibody; however, the level of BLM protein in the FANCF null extract input was dramatically reduced as detected by western blot analysis (see next section), making it technically difficult to perform the experiment.

Since FANCF and BLM both interact with DNA, we tested if their association was DNA dependent. HeLa NEs were pretreated with either DNase I or ethidium bromide (EtBr), and subsequently incubated with anti-FANCF antibody. Western blot analysis of the immunoprecipitates demonstrated that both BLM and FANCF were reciprocally co-immunoprecipitated (Figure 1C). Thus, the association of the FANCF and BLM helicases in NEs is resistant to DNA degradation by DNase I or EtBr intercalation, suggesting that their interaction is not dependent on DNA.

Since FANCF and BLM are known to respond to interstrand cross-links and replication stress, we examined if their interaction was affected when cells are exposed to MMC or hydroxyurea (HU). Results from experiments using either anti-BLM or anti-FANCF antibodies demonstrated that their interaction was increased approximately two-fold after cellular exposure to 50 nM MMC or 3 mM HU (Figure 1D).

FANCF and BLM interact directly

To determine if FANCF and BLM interact directly, we performed co-immunoprecipitation experiments with purified recombinant FANCF and BLM proteins. Using antibodies directed against either FANCF or BLM, the two helicase proteins were reciprocally co-immunoprecipitated (Figure 1E, lanes 4 and 7). In control experiments, neither FANCF nor BLM was precipitated when primary antibody was omitted or when normal rabbit IgG was used (Figure 1E, lanes 5, 6, 8, and 9). Moreover, FANCF antibody failed to precipitate BLM when FANCF was absent from the binding mixture. Likewise, BLM antibody failed to precipitate FANCF when BLM was absent from the binding mixture (data not shown). The co-immunoprecipitation of purified BLM and FANCF proteins was resistant to DNase I or EtBr, suggesting that the proteins can interact independently of DNA (Supplementary Figure S1A).

To confirm that FANCF and BLM interact directly with each other, enzyme-linked immunoabsorbent assay was used to test for the protein interaction (Supplementary Figure S1B). FANCF was incubated in the presence of 3% bovine serum albumin (BSA) with BLM immobilized on microtiter wells, and the bound FANCF protein was detected immunologically. The colorimetric signal was dependent on FANCF protein concentration (Supplementary Figure S1C). Specific binding of FANCF to BLM was analysed according to Scatchard-binding theory. Data were analysed by a Hill plot and found to be linear, indicating a single set of binding sites for FANCF with BLM. The apparent dissociation (K_d) was determined to be 4 nM. The colorimetric signal from FANCF binding to immobilized BLM was specific since a significantly reduced signal was observed in wells precoated with BSA (Supplementary Figure S1C). The colorimetric signal from the FANCF–BLM interaction was resistant to pretreatment of both FANCF protein and BLM with DNase I (2 µg/ml) (Supplementary Figure S1C), suggesting that a contaminating DNA bridge is not responsible for the signal.

The C-terminal domain of FANCF binds to BLM

To define the domain on FANCF required for BLM binding, several FANCF-myc fusion proteins of varying length were expressed in 293T cells, and the corresponding FANCF protein fragments were precipitated from the cell lysates with c-Myc antibody tagged agarose beads (Figure 1F). Full-length FANCF

Figure 1 FANCI and BLM are reciprocally co-immunoprecipitated from human nuclear extracts and the C-terminal domain of FANCI binds BLM. (A) FANCI antibody (top) or BLM antibody (bottom) was used to co-immunoprecipitate FANCI and BLM from HeLa NE (nuclear extract). The blot was probed with antibodies against indicated proteins. HeLa NE (lane 2) represents 5% of the input. (B) BLM antibody (top) or FANCI antibody (bottom) was used to co-immunoprecipitate BLM and FANCI from NE of BLM null (BLM^{-/-}) or BLM corrected (BLM^{+/+}) cells. Blots were probed as described above. (C) FANCI antibody co-immunoprecipitates FANCI and BLM from HeLa NE in the presence of ethidium bromide (10 µg/ml) or DNase I (2 µg/ml). The blot was probed with goat anti-BLM (top), rabbit anti-FANCI (middle), and mouse anti BRCA1 (bottom) antibodies. (D) Whole-cell extracts of HeLa cells that were prepared from untreated or treated with MMC (50 nM) or HU (3 mM) and immunoprecipitated with anti-FANCI or anti-BLM antibody as indicated. The blots were probed with indicated antibodies. (E) Purified FANCI and BLM proteins were reciprocally co-immunoprecipitated from a mixture of the two proteins using antibodies against FANCI or BLM. The blot was probed with anti-BLM (top) or anti-FANCI (bottom) antibodies. (F) The different FANCI constructs are indicated with a positive (+) or negative (–) to indicate binding to BLM. (G) Myc IP experiments were performed from 293T cells that were transfected with vector alone, full-length FANCI or the specified FANCI construct. IP products were analysed by western blot with Myc or BLM antibodies. The asterisk denotes the migration of the different myc-tagged FANCI species.

and FANCI expression construct FANCI_{1–900} precipitated BLM, but FANCI_{1–660} did not, suggesting that the FANCI N-terminal residues 1–660 are not required for BLM binding (Figure 1G). Expression of C-terminal FANCI fragments FANCI_{881–1249} and FANCI_{881–1060} were sufficient to co-precipitate BLM, suggesting that a BLM interaction site resides in a C-terminal non-catalytic domain of FANCI spanning residues 881–1060 (Figure 1G).

Cellular BLM protein levels are strongly dependent on FANCI status

Since the evidence suggested that BLM and FANCI interact with each other and may exist in a complex *in vivo*, we wanted to assess if their respective protein stabilities might be affected by each other. To address this possibility, we performed western blot analyses of BLM and FANCI in either FA-J mutant cells or HeLa cells that had been depleted of

Figure 2 Cellular BLM protein levels are strongly dependent on FANCI status. (A) NE was prepared from HeLa, FA-J, siRNA-control-treated HeLa, and siRNA-FANCI-treated HeLa cells, and proteins were transblotted and probed with indicated antibodies. (B) HeLa cells were transfected with siRNA-FANCI or siRNA-control and harvested at indicated time points after a single transfection. Whole-cell extracts were prepared as described in Materials and methods. (C) RT-PCR experiments were performed as described in Materials and methods. Experimental data represent the mean of three independent experiments in triplicate with standard deviations indicated by error bars. (D) NE was prepared from FA-J mutant and FA-J corrected cells, and proteins were transblotted and probed with indicated antibodies. (E) Western blot showing FANCI or BLM levels of either control or FANCI-siRNA transfected HeLa cells treated with indicated concentrations of MG132 for 6 h. Untreated cells were subjected to DMSO treatment alone. (F) NE was prepared from HeLa, BLM^{-/-}, and BLM^{+/-} cells, and proteins were transblotted and probed with anti-BLM and anti-FANCI antibodies. For all western blots, β-actin served as a loading control.

FANCI protein using RNA interference. BLM protein levels were dramatically decreased by 80–90% in FA-J cells or HeLa cells that had been depleted of FANCI (Figure 2A). In control experiments, WRN protein levels were not significantly reduced in the FA-J mutant cells or in cells that had been depleted of FANCI (Figure 2A). Time course experiments

demonstrated that BLM protein levels declined within 24 h after a single siRNA-FANCI transfection in HeLa cells (Figure 2B).

The significant reduction in BLM protein levels in FANCI-depleted cells raised the possibility that BLM transcript abundance was reduced. Real-time PCR analysis of BLM

and FANCD2 transcript levels were performed using siRNA-FANCD2 and siRNA-control transfected HeLa cells. The relative BLM transcript levels were the same in FANCD2-depleted and control cells (Figure 2C), indicating that the effect of FANCD2 depletion on BLM protein levels was not manifested at the transcription level.

To confirm that the cellular level of BLM protein is dependent on FANCD2 status, we examined BLM protein levels in an isogenic pair of FANCD2 mutant and corrected cell lines. As shown in Figure 2D, BLM protein levels were significantly reduced in the FA-J mutant cells compared with the same FANCD2 corrected cells. Since BLM is known to interact with TopoII α (Wu *et al*, 2000; Johnson *et al*, 2000; Hu *et al*, 2001), RPA70 (Brosh *et al*, 2000), and FEN-1 (Sharma *et al*, 2003), we examined the levels of these BLM-interacting proteins in the FA-J mutant and corrected isogenic pair of cell lines. TopoII α , RPA70, and FEN-1 protein levels were comparable in the FA-J mutant and corrected cell lines (Figure 2D), suggesting that the effect of FANCD2 status on BLM protein level is highly specific.

To better understand the effect of FANCD2 status on BLM protein stability, we examined the effect of the proteasome inhibitor MG132 on BLM protein level in FANCD2-depleted or mock-treated cells. In these experiments, HeLa cells transfected with control or FANCD2 siRNA were subsequently treated with the indicated concentrations of MG132 for 6 h. Whole-cell lysates were evaluated by western blot for FANCD2 and BLM protein. In FANCD2-depleted cells, BLM protein was restored in cells exposed to 10 μ M MG132 (Figure 2E). These results suggest that BLM protein is degraded by a proteasome-mediated pathway in FANCD2-deficient cells.

Next, we examined FANCD2 protein levels in an isogenic pair of BLM mutant and corrected cell lines. As shown in Figure 2F, FANCD2 protein amounts remained comparable in the BLM mutant and corrected cells, indicating that cellular FANCD2 protein levels are not affected by BLM status.

Interaction of FANCD2 and BLM remains intact in FANCA, FANCD2, or BRCA1-deficient cells

We next asked whether the FANCD2–BLM interaction was lost in FA-deficient cells. Western blot analysis of NEs showed that BLM protein levels were reduced in FA-A (Figure 3A, lane 2 compared with lane 1) or FA-D2 (Figure 3A, lane 4 compared with lane 3) mutant cell lines compared with the corrected cells. FANCD2 and BLM could be co-immunoprecipitated from NE of the FA-A or FA-D2 mutant cell lines (Figure 3A), suggesting that FANCD2 and BLM are associated with each other in cells that are defective in either a member of the FA core complex (FANCA) or FANCD2.

Since FANCD2 is known to interact with BRCA1 (Cantor *et al*, 2001), we asked if the FANCD2–BLM interaction would remain intact in BRCA1-deficient cells. Co-immunoprecipitation experiments were performed using BRCA1 mutant and corrected cells. FANCD2 and BLM were reciprocally co-immunoprecipitated from BRCA1 mutant or corrected cells (Figure 3B), suggesting that the FANCD2–BLM interaction is not dependent on BRCA1. Furthermore, since the binding site for BLM on FANCD2 overlaps that of BRCA1, we sought to determine if a FANCD2-S990A mutant defective in the BRCA1 interaction would still be able to bind BLM. The results from anti-FANCD2 immunoprecipitation experiments using extracts from transfected FA-J cell lines expressing FANCD2-S990A or FANCD2-WT demonstrated that BLM was co-immunoprecipitated with either FANCD2 protein equally well, whereas BRCA1 failed to be co-immunoprecipitated with FANCD2-S990A (Supplementary Figure S2A).

The BRCA1-associated complex (BASC) identified by Wang *et al* (2000) contains BRCA1 and BLM, as well as BLM-interacting partners MLH1 and RPA. To determine if proteins found in the BASC complex associate with BRCA1 through FANCD2, anti-BRCA1 co-immunoprecipitation experiments were performed with extracts from FA-J null and corrected cells. MLH1, RPA, and FANCA were all

Figure 3 The interaction between BLM and FANCD2 helicases is not dependent on the integrity of the FA core complex, FANCD2, or BRCA1. (A) Immunoprecipitate from NE of the indicated FA mutant or corrected cell line using goat anti-BLM antibody (top panel) or rabbit anti-FANCD2 antibody (middle panel) was analysed by western blotting using anti-FANCD2 and anti-BLM antibodies. Input (bottom panel) represents 5% of NE input for co-immunoprecipitation experiments. (B) Immunoprecipitate from NE of BRCA1 mutant, or BRCA1 corrected cell lines using anti-FANCD2 antibody (top panel) or goat anti-BLM antibody (bottom panel) was analysed by western blotting using anti-FANCD2 and anti-BLM antibodies.

Figure 4 FANCI and BLM foci formation after cellular exposure to the replication inhibitor hydroxyurea. HeLa cells were either left untreated or treated with HU (2 mM) as described in Materials and methods. After fixation and permeabilization, cells were stained with anti-FANCI (red) or anti-BLM (green) antibodies. After treatment with HU, BLM partially co-localizes in nuclear foci that coincide with FANCI foci as shown in the merged images.

co-immunoprecipitated with BRCA1 in the FA-J null cells similar to FA-J corrected cells (Supplementary Figure S2B).

To determine if the interaction of the BLM-interacting protein TopoIII α with the FA core complex (Deans and West, 2009) was altered when FANCI was absent, anti-FANCI co-immunoprecipitation experiments were performed with extracts from FA-J null and corrected cells. TopoIII α was co-immunoprecipitated with FANCI in the FA-J null cells similar to FA-J corrected cells (Supplementary Figure S2C).

Induction of FANCI and BLM foci following replication stress

Evidence suggests that both BLM (Bachrati and Hickson, 2008) and FANCI (Wu *et al*, 2008; Hiom, 2010) are important for the cellular response to replication stress. To address the possibility that the two helicases become associated with each other in subnuclear foci after replication stalling, we examined the co-localization of endogenous BLM and FANCI by immunofluorescence after exposure of HeLa cells to the replication inhibitor HU. Both BLM and FANCI formed distinct nuclear foci after HU treatment (Figure 4; Supplementary Figure S3), a result consistent with earlier published data (Davies *et al*, 2004; Pichierri *et al*, 2004). Furthermore, BLM and FANCI foci partially co-localized after replication stress imposed by HU (Figure 4; Supplementary Figure S3). FANCI and BLM foci also partially co-localized in HeLa cells exposed to MMC (50 ng/ml) or ionizing radiation (IR; 10 Gy); however, the number and intensity of BLM and FANCI foci formed was not as great in the case of IR treatment (data not shown). The stabilizing effect of FANCI on BLM protein levels may help maintain independent recruitment of BLM to stalled forks or damaged sites.

Although BLM protein levels are significantly reduced in FANCI null or FANCI-depleted cells, the residual BLM protein in FANCI-deficient cells formed nuclear foci after DNA damage (MMC, IR) or replication stress (HU) (Supplementary Figure S4A and B). FANCI foci formed after HU, MMC, or IR in BLM null cells (Supplementary Figure S4C). These results suggest that BLM or FANCI foci are able to form after DNA damage or replication stress in the absence of the interacting helicase partner.

FANCI-depleted cells are sensitive to replication stress

We next sought to determine if FANCI-deficient cells, like BLM-deficient cells, are sensitive to replication stress. To do this, FANCI-depleted and control-siRNA HeLa cells (Figure 5A) were exposed to increasing concentrations of HU and cell proliferation was evaluated by the WST-1 assay as a measure of metabolic activity. FANCI-depleted cells were significantly more sensitive to all HU concentrations analysed (Figure 5B). As a control, FANCI-depleted cells were also more sensitive to the DNA cross-linking agent MMC compared with siRNA-control cells (Supplementary Figure S5). We also examined HU sensitivity in cells depleted of FANCI that were deficient in BLM (PSNG13) compared with BLM-proficient cells (PSNF5) (Figure 5C and D). PSNF5 cells depleted of FANCI displayed reduced BLM protein level and were more sensitive to HU compared with control-siRNA PSNF5 cells (Figure 5D), a result that was consistent with FANCI depletion studies in HeLa cells (Figure 5B). However, FANCI-depleted PSNG13 cells were more HU resistant than control-siRNA PSNG13 cells (Figure 5D), suggesting that the presence of FANCI in a BLM-deficient background is toxic to the cells during replication stress.

The HU sensitivity of FANCI-deficient cells may be due to a reduced level of BLM. Alternatively, FANCI may have a more direct role in HU resistance. To address the importance of FANCI catalytic activity in HU resistance, FA-J cells were transfected with a plasmid expressing the helicase-inactive FANCI-K52R Walker A box mutant protein (Cantor *et al*, 2004) and HU resistance of these cells was compared with FA-J cells expressing FANCI-WT or transfected with empty vector (Figure 5). As shown in Figure 5E, expression of FANCI-K52R in FA-J cells restored BLM protein levels similar to FA-J cells expressing FANCI-WT; moreover, HU resistance was partially but not fully restored (Figure 5F). These results suggest that FANCI ATPase/helicase activity is required for complete HU resistance.

Domain mapping experiments demonstrated that BLM interacts with the C-terminal region, but not the N-terminal region, of FANCI (Figure 1F and G). To determine if the physical interaction of BLM with FANCI is important for HU resistance, Myc-tagged FANCI fragments were expressed in 293T cells (Figure 6A). The transfected cell lines were

Figure 5 FANCI-depleted cells are sensitive to replication stress. HeLa cells were transfected twice with either control or FANCI siRNA as described in Materials and methods and evaluated for FANCI protein level with β -actin serving as a loading control (**A**). One day after the second siRNA transfection, cells were exposed to the indicated concentrations of HU (**B**). Cell proliferation was measured using the WST-1 assay (Roche, Indianapolis, IN) after 48 h. (**C**, **D**) PSNG13 (BLM $^{-/-}$) or PSNF5 (BLM $+/+$) cells were transfected twice with either control or FANCI siRNA as described under Experimental Procedures and evaluated for FANCI protein level with β -actin serving as a loading control (**C**). One day after the second siRNA transfection, cells were exposed to the indicated concentrations of HU (**D**). Cell proliferation was measured using the WST-1 assay after 48 h. (**E**, **F**) FA-J cells transfected with vector alone, wild-type FANCI, or FANCI-K52R were evaluated for expression of FANCI/FANCI-K52R protein (**E**). The transfected cells were exposed to the indicated concentrations of HU and cell proliferation was measured using the WST-1 assay after 48 h (**F**). Experimental data are the mean of three independent experiments done in triplicate and s.d. indicated by error bars.

tested for HU sensitivity and co-immunoprecipitation of endogenous FANCI and BLM. 293T cells expressing the BLM-interacting Myc-FANCI₈₈₁₋₁₂₄₉ or Myc-FANCI₈₈₁₋₁₀₆₀ were found to be sensitive to HU compared with cells expressing Myc-FANCI₁₋₄₉₉ or transfected with empty vector

expressing just the Myc epitope (Figure 6B). To determine if expression of FANCI fragments affected the interaction of endogenous FANCI with BLM, anti-BLM co-immunoprecipitation experiments were performed. The FANCI-BLM co-immunoprecipitation was decreased by 2.5-fold for extracts

Figure 6 BLM-interacting fragments of FANCI expressed in 293T cells disrupt the FANCI-BLM interaction and lead to HU sensitivity. 293T cells transfected with vector or indicated FANCI constructs were evaluated for their expression (**A**). The transfected cells were treated with the indicated concentrations of HU. Cell proliferation was measured using the WST-1 assay after 48 h (**B**). Experimental data are the average of three independent experiments done in triplicate and s.d. indicated by error bars. (**C**) Whole-cell lysates prepared from 293T cells transfected with either vector or indicated FANCI constructs were used for immunoprecipitation with anti-BLM antibody. The immunoprecipitate was analysed by western blot using anti-BLM or anti-FANCI antibody. Whole-cell lysate from transfected 293T cells represents 5% of the input used for the co-immunoprecipitation.

from cells expressing Myc-FANCI₈₈₁₋₁₀₆₀ and 3.1-fold for Myc-FANCI₈₈₁₋₁₂₄₉ (Figure 6C). These results suggest that expression of a C-terminal fragment of FANCI that interacts with BLM exerts a dominant negative effect on HU resistance by interfering with the FANCI-BLM interaction.

Increased SCE in FA-J cells

A hallmark of BS is elevated SCE, raising the possibility that FANCI mutated cells may display a similar form of chromosomal instability. Chromosomal spreads from wild-type, FA-J, and FA-C fibroblasts were prepared and analysed for SCE. FA-J fibroblasts displayed a significantly greater level of SCE compared with the wild-type cells (Table I). In contrast, FA-C cells displayed a level of SCE that was comparable to wild-type cells.

To assess the effect of a combined BLM and FANCI deficiency on SCE, FANCI was depleted from an isogenic pair of BLM null and BLM corrected cells. The results from these assays demonstrated that FANCI depletion in a BLM null background did not increase SCE frequency (Supplementary Table 1). However, FANCI depletion in a BLM wild-type background increased SCE frequency (Supplementary Table 1), a result that was consistent with what was observed for FA-J cells (Table I). These results

Table I Quantification of SCE frequency

Cell line	SCE/chromosome						SCEs	Chromosomes	Mean
	0	1	2	3	4	5			
Wild type	772	233	42	4			329	1051	0.31
FANCI	527	296	88	28	4	1	562	944	0.59
FANCC	685	198	37	3	1		285	924	0.31

χ^2 FANCI versus WT $P < 0.0001$.

suggest that acutely depleting FANCI in a BLM-deficient background does not further elevate SCE frequency.

Functional interaction of BLM and FANCI helicases

The physical interaction between FANCI and BLM raised the possibility that the two helicases might functionally interact through their DNA unwinding functions. To test this, a mixture of purified FANCI and BLM helicases was evaluated for their ability to unwind DNA substrates and compared with unwinding reactions performed using either helicase alone. FANCI and BLM unwinding of a simple forked duplex substrate was additive (Supplementary Figure S6), suggesting

that BLM and FANCI do not synergistically interact on an undamaged forked duplex substrate.

Previously, we reported that FANCI helicase activity was profoundly sensitive to a polyglycol backbone modification in either the translocating or non-translocating strands of the duplex, suggesting that FANCI interactions with the sugar phosphate backbone of *both* strands of the duplex are important as FANCI tracks along the DNA molecule and separates the strands (Gupta *et al*, 2006). To test if FANCI and BLM might work together to unwind the forked duplex with the backbone discontinuity, we performed experiments similar to those described for the undamaged forked duplex, except that the substrate contained a polyglycol linkage within the duplex in either the bottom (BLM translocating) or top (FANCI translocating) strands (Figure 7A and C). BLM helicase alone failed to unwind the forked duplex substrate containing a polyglycol linkage in the bottom strand along which it translocates (Figure 7A); however, BLM was able to unwind the same forked duplex substrate when the polyglycol linkage was positioned in the top strand opposite to the one that BLM translocates along (Figure 7B). In contrast, FANCI poorly unwound the DNA substrate with a polyglycol

substrate in either the top (Figure 7A) or bottom (Figure 7B) strands, consistent with our previous observations (Gupta *et al*, 2006). These results suggest that FANCI is uniquely sensitive to the backbone disruption and distinguishes its behaviour from that of the human RecQ helicase BLM.

We next asked if the combined presence of BLM and FANCI might result in a synergistic unwinding of the damaged DNA substrates. As shown in Figure 7A, a mixture of the FANCI and BLM helicases completely failed to unwind the forked duplex substrate harbouring the polyglycol linkage in the bottom, BLM translocating, strand. However, incubation of both BLM and FANCI helicases with the forked duplex harbouring the polyglycol linkage in the top (FANCI translocating) strand resulted in a significantly increased level of unwinding throughout the BLM titration (1.2–10 nM) (Figure 7B, and shown quantitatively in Figure 7C). It was conceivable that FANCI binding to the fork rather than its helicase activity was responsible for maximal promotion of BLM helicase activity on the damaged DNA substrate. However, results from BLM helicase assays on this substrate in the presence of the T4 helicase loading protein gp59 that preferentially binds forked DNA structures (Mueser *et al*,

Figure 7 FANCI and BLM helicases together more efficiently unwind a damaged DNA substrate with sugar phosphate backbone discontinuity. Helicase assays were performed as described under Materials and methods using the indicated concentrations of BLM and FANCI helicases and forked duplexes of 31 bp, which contained a polyglycol linkage in the sugar phosphate backbone in either the bottom strand (**A**) or top strand (**B**) as indicated. Filled triangle, heat-denatured DNA substrate control. (**C**) The quantitative analysis of unwinding data on the substrate with backbone discontinuity in the top strand (**B**) in the presence of BLM alone or BLM with FANCI-WT or FANCI-K52R. Note that FANCI alone poorly unwound the forked duplex substrate with backbone discontinuity in either the top or bottom strands (lane 10). Data represent the mean of at least three independent experiments with standard deviations indicated by error bars. (**D**, **E**) The quantitative analysis of WRN and RECQ1 helicase reactions, respectively, in the presence of FANCI (open circle) or the absence of FANCI (filled circle) conducted with the 31-bp forked duplex that had a polyglycol linkage in the top strand.

2000) demonstrated that BLM unwinding activity was not stimulated (Supplementary Figure S7), suggesting that the effect is not attributed to simply forked DNA substrate binding by the FANCI protein. Incubation of BLM and a helicase-inactive FANCI-K52R mutant with the forked duplex substrate containing the polyglycol linkage in the top strand yielded a greater level of unwinding throughout the BLM titration as well; however, the unwinding at a given BLM concentration was not as high as the level observed when BLM and FANCI-WT was incubated with the damaged DNA substrate (Figure 7C). FANCI-K52R did not stimulate BLM helicase activity on the DNA substrate with the polyglycol linkage in the bottom strand (data not shown). These results demonstrate that under these reaction conditions, FANCI can stimulate BLM helicase activity on a DNA substrate with a backbone discontinuity in the FANCI translocating strand.

To determine if the interaction between FANCI and BLM helicases was specific, we tested the effect of the human RecQ helicases WRN and RECQ1 on the ability of FANCI to unwind the forked duplex substrate with the polyglycol linkage in the top strand. For these substrates, both RECQ1 and WRN behave like BLM, and are inhibited by the polyglycol linkage in a strand-specific manner, that is, RECQ1 and WRN fail to unwind the forked duplex substrate when the polyglycol linkage is located within the duplex in the translocating (bottom) strand (manuscript in preparation). Incubation of either WRN (Figure 7D) or RECQ1 (Figure 7E) and FANCI helicases with the forked duplex harbouring the polyglycol linkage in the top, FANCI translocating, strand resulted in little or no increase in DNA unwinding compared with WRN or RECQ1 alone. These results suggest that WRN or RECQ1 do not interact with FANCI in a manner to unwind the damaged DNA substrate as observed in reaction mixtures containing BLM.

Discussion

In this study, we report that the FANCI and BLM helicases can be reciprocally co-immunoprecipitated from human NEs, and that their association is not dependent on the integrity of the FA core complex, FANCD2, or BRCA1. We observed that the FANCI–BLM interaction is enriched when cells are exposed to HU or DNA damaging agents and the helicases co-localize in nuclear foci after HU, suggesting that they operate together in a pathway responsible for helping cells cope with replication stress. Indeed, we show that FANCI-deficient cells, like BLM-deficient cells, are sensitive to the replication inhibitor HU and show elevated SCE. FANCI status profoundly influences cellular BLM protein levels, suggesting their direct physical interaction *in vivo*. In support of this, biochemical interaction assays with purified recombinant proteins demonstrated that BLM and FANCI helicases directly bind to each other in a manner that is not dependent on DNA. Thus, BLM and FANCI interact with each other *in vitro* and *in vivo*.

One of the most striking observations from our studies is that BLM protein levels are significantly reduced in FANCI null cells or in cells that are acutely depleted of FANCI using RNA interference. Although the BLM protein level was markedly reduced in FANCI-deficient cells, the levels of interacting partners of BLM (RPA, FEN-1, TopoIII α) were not reduced, suggesting that the absence or reduction of intracellular FANCI protein concentration exerted an effect specifically

on BLM. Presumably, the physical interaction between BLM and FANCI helps to stabilize the BLM protein *in vivo*; however, the converse is not true, that is, the FANCI protein level was not different in BLM mutant and corrected cells. The observed decline in BLM protein level in FANCI-deficient cells suggests that cellular phenotypes associated with FANCI deficiency may at least partly reflect an underlying reduction in BLM protein abundance. Interestingly, BLM protein was also reduced in FA-A or FA-D2 cell lines. BLM protein levels are known to be affected when certain interacting partners (e.g., RMI1 (Yin *et al*, 2005) and RMI2 (Xu *et al*, 2008)) are deficient, suggesting that BLM folding or susceptibility to proteasome degradation is highly influenced by interacting/associated proteins in the FA or related pathways. Both BS cells (Pichierri *et al*, 2004) and FA-J cells are hypersensitive to DNA cross-linking agents (Litman *et al*, 2005; Peng *et al*, 2007). A hallmark of BS is elevated SCE (Hanada and Hickson, 2007), also detected in chicken *brip1* cells (Bridge *et al*, 2005) and, as shown here, in FANCI-deficient human cells. Although the ~2-fold SCE increase in FA-J cells was not as great as the 7- to 10-fold increase observed in cells from BS patients (Chaganti *et al*, 1974; German, 1993), it is comparable to that observed following depletion of BLM (Yin *et al*, 2005) or FANCM (Deans and West, 2009). In human cells, FANCI knockdown by RNA interference resulted in elevated MMC-induced chromosomal abnormalities including breakage, chromatid interchanges, triradials, and quadriradials (Bridge *et al*, 2005; Litman *et al*, 2005), a chromosomal structure typically observed in BS patients (van Brabant *et al*, 2000). Hence, the chromosomal instability associated with FANCI deficiency may at least partly reflect impaired BLM function in FA-J null cells.

The fact that DNA damage-induced FANCD2 monoubiquitination is intact in FANCI-deficient cells (Litman *et al*, 2005) suggests that FANCI helicase functions downstream in the FA pathway. Similarly, BLM is not required for FANCD2 monoubiquitination or focus formation (Pichierri *et al*, 2004), suggesting a downstream role as well. The FA core complex is required for BLM phosphorylation following treatment with a DNA cross-linker but not HU (Pichierri *et al*, 2004), implicating the FA core complex as an upstream regulator of BLM function by setting the stage for homologous recombination repair at blocked replication forks. Moreover, Hirano *et al* (2005) reported that MMC-induced formation of GFP-BLM foci was dramatically reduced in both human and chicken *fancc* or *fancd2* cells. A functional linkage between FANCC and BLM was proposed since the level of SCE in chicken *blm* cells was similar to that of *fancc/blm* cells (Hirano *et al*, 2005). Cross-talk between BLM and proteins of the FA pathway is supported by experimental evidence that the FA core protein complex and the BLM complex associate together in a supercomplex known as BRAFT (BLM, RPA, FA, and TopoIII α) (Meetei *et al*, 2003). Although FANCI was not identified by mass spec analysis of BLM-interacting proteins from unstressed cells, it is possible that the interaction of FANCI with BLM is transient and less stable than that of the BRAFT complex or that FANCI and BLM exist in a protein complex that is regulated following cellular stress and is distinct from the BRAFT complex. In subsequent work, Singh *et al* (2008) identified FANCM in complexes containing BLM and RMI2, a novel factor required for the stability of the BLM complex and also important for chromosomal stability

(Singh *et al*, 2008; Xu *et al*, 2008). Our results demonstrate that BLM interacts with FANCIJ in addition to the FA core complex. Since BLM, but not TopoIII α , is depleted in FANCIJ-deficient cells, it is possible that FANCIJ regulates a very specific subset of BLM reactions (e.g. fork regression) not requiring the topoisomerase.

BLM co-localizes with FANCIJ (this study) and FANCD2 (Pichierri *et al*, 2004) in response to cellular exposure to HU or MMC. Therefore, it is likely that BLM, FANCIJ, and monoubiquitylated FANCD2 preferentially associate with each other at stalled or blocked replication forks. The single-stranded DNA-binding protein RPA, which interacts physically and functionally with both BLM and FANCIJ, strongly co-localizes with FANCIJ after replication stress as well (Gupta *et al*, 2007). The concerted action of BLM and FANCIJ with their protein partners such as RPA and FANCD2 is likely to be important for their collective role in the recovery from S-phase progression and maintenance of genomic stability. The observations that FANCD2 monoubiquitination is required for its association with chromatin and subnuclear localization with BRCA1, RAD51, MRE11-RAD50-NBS1, RPA, PCNA, and BRCA2 (Gurtan and D'Andrea, 2006), together with the current results, suggest a scenario in which the critical DNA repair steps of the FA pathway are orchestrated by FANCD2 and enacted by the FANCIJ and BLM helicases with HR and DNA repair proteins.

The concerted action of FANCIJ and BLM helicases to unwind a DNA structure associated with a stalled replication fork or HR repair may enable subsequent processing by structure-specific nucleases to ensure genome integrity or serve to clear proteins bound to DNA (e.g. Rad51; Bugreev *et al*, 2007; Sommers *et al*, 2009) to complete recombinational repair. Both BLM and FANCIJ recognize and unwind a D-loop structure (Gupta *et al*, 2005; Bachrati *et al*, 2006), an early intermediate of HR. However, only BLM unwinds a Holliday Junction structure (Karow *et al*, 2000; Mohaghegh *et al*, 2001; Gupta *et al*, 2005), and FANCIJ does not stimulate BLM unwinding of this late HR intermediate (our unpublished data).

The functional interaction between FANCIJ and BLM on the DNA substrate with a polyglycol linkage in the BLM translocating strand suggests that the helicase interaction may be important for traversing backbone damage. The ability of FANCIJ to interact with BLM may facilitate unwinding past the lesion in a specific manner, since DNA unwinding by WRN or RECQ1 on the same substrate was not increased by the presence of FANCIJ. The ability of the catalytically inactive FANCIJ-K52R mutant protein to stimulate BLM helicase activity suggests that the protein interaction is an important factor in the functional interaction. However, further mechanistic studies are necessary to understand the importance of the physical interaction between BLM and FANCIJ for DNA unwinding by the two helicases acting together perhaps in a protein complex. A paradigm for two helicases with opposite polarities functioning together was provided by the molecular characterization of the RecBCD complex in which RecB and RecD helicases, which translocate in opposite directions along ssDNA, unwind duplex DNA in the same direction by moving along complementary strands of the Watson-Crick DNA double helix (Singleton *et al*, 2004).

Although BLM and FANCIJ interact, the distinctive clinical features of BS and FA group J patients indicate that the two

helicases also have independent roles. It will be important to quantify BLM protein levels in individuals with FANCIJ mutations and relate this to their clinical symptoms. According to the Fanconi Anemia Mutation Database maintained by The Rockefeller University, FANCIJ protein variants exist that fall into three classes: substitutions, frameshifts, and nonsense mutations. Clinical heterogeneity in FA-J patients may be dependent on the nature of the FANCIJ mutation as it relates to the stability of the dysfunctional FANCIJ protein it encodes. For example, western blot analysis of FA-J patient cell lines demonstrated the absence of full-length FANCIJ protein in cells harbouring frameshift, nonsense, or deletion mutations, but residual full-length FANCIJ protein in a cell line harbouring a FANCIJ missense mutation (Levitus *et al*, 2005). The interaction of FANCIJ with BLM and the strong effect of FANCIJ deficiency on BLM protein stability suggest that FA-J patient cell lines should be screened for BLM protein by western blot analysis. BLM deficiency in FA-J patients may be a contributory factor to the nature and degree of chromosomal instability, cancer, and associated phenotypes observed in the individuals.

Materials and methods

Proteins

Baculoviruses encoding FANCIJ or FANCIJ-K52R with a C-terminal FLAG tag were used to infect high five insect cells and the recombinant FANCIJ protein was purified as described previously (Cantor *et al*, 2004). Recombinant WRN (Sharma *et al*, 2004), RECQ1 (Sharma *et al*, 2005), and BLM (Karow *et al*, 1999) proteins were purified as described previously. Purified gene 59 protein was a kind gift from Dr Stephen Benkovic (Pennsylvania State University).

Cell lines

HeLa, FA-J (EUFA30-F), and 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% l-glutamine at 37°C in 5% CO₂. FA-J cells infected with the pOZ retroviral vector containing no insert or wild-type FANCIJ were prepared as described (Peng *et al*, 2007). BLM mutant (PSNG13) and BLM corrected (PSNF5) were grown in the same media but supplemented with 350 µg/ml G418. Immortalized fibroblasts FA-A (PD220), FA-D2, and their respective corrected cells, provided by the Fanconi Anemia Research Fund, were grown in the same medium as HeLa but supplemented with 0.2 mg/ml puromycin. HCC1937, which carries a hypomorphic mutation in BRCA1, and the corresponding BRCA1-reconstituted cells were grown in RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin.

For FANCIJ knockdown, FANCIJ siRNA (100 nM; Dharmacon, Chicago, IL) was transfected into HeLa cells using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. After two transfections on consecutive days, cells were harvested 48 h after the second transfection.

GFP-vector or GFP-FANCIJ plasmid DNA (2 µg) was used for transfecting BLM null and BLM corrected cells using Lipofectamine-2000 (Invitrogen). FA-J null cells were transfected using the Nucleofector Kit (Lonza) with plasmid DNA encoding Myc-FANCIJ-WT, Myc-FANCIJ-S990A, or vector according to the manufacturer's protocol.

MG132 (Sigma, St Louis, MO) was dissolved in DMSO (10 mM) and small aliquots (30 µl) were stored at -80°C. HeLa cells transfected with control or FANCIJ siRNA were subsequently treated with the indicated concentrations of MG132 for 6 h. Untreated cells were subjected to DMSO treatment alone.

SCE analysis

Cells were incubated with 10 µM BrdU for two cell cycles and treated with 0.1 µg/ml Colcemid for the last 2 h. After harvesting, cells were incubated in 0.075 M KCl solution for 20 min, and subsequently fixed in methanol and acetic acid (3:1 ratio) for 30 min. Cells were dropped onto 50%-ethanol-wet slides and dried.

Slides were then incubated with 10 µg/ml Hoechst 33258 in phosphate buffer (pH 6.8) for 20 min. Slides were exposed to 352 nm black light for 30–60 min, followed by incubation in 2 × SSC at 62°C for 1 h. They were then stained using 3% Giemsa solution (pH 6.8) for 10 min.

Co-immunoprecipitation experiments and western blot analyses

NEs were prepared from exponentially growing HeLa, FA-A, and FA-D2 cells as described previously (Dignam *et al*, 1983).

NE (1 mg protein) was incubated with rabbit anti-FANCI polyclonal antibody (1 µg; Sigma), goat anti-BLM (1 µg C-18; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-BRCA1 (1 µg 9010; Cell Signaling Technology, Danvers, MA; rabbit anti-FANCA (kindly provided by Dr Weidong Wang, NIA-NIH), normal rabbit IgG antibody or normal goat IgG antibody (1 µg, Santa Cruz Biotechnology) in Buffer D (50 mM HEPES (pH 7.5), 100 mM KCl, 10% glycerol) for 2 h, and subsequently mixed with 20 µl protein-G agarose (Roche) for 2 h at 4°C. Beads were washed with Buffer D containing 0.1% Tween-20. Immunoprecipitations from FA mutant and BRCA1 null cell lines were performed using whole-cell lysates prepared as described previously (Gupta *et al*, 2007). Proteins were eluted by boiling in SDS sample buffer, resolved on 10% polyacrylamide Tris-glycine SDS gels, and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were probed for FANCI or BLM using the rabbit polyclonal anti-FANCI (1:2000) or goat anti-BLM (1:500) antibodies, respectively. Proteins on immunoblots were detected using ECL (Amersham Biosciences).

Co-immunoprecipitation experiments using purified proteins were performed by incubating purified FANCI (100 ng) and purified BLM (100 ng) proteins together at 4°C for 2 h. This binding mixture was used as the input for immunoprecipitation experiments, as described above.

For FANCI mapping studies, plasmids encoding Myc-FANCI fragments were used to transfect 293T cells as described previously (Peng *et al*, 2007). 293T cells were harvested and lysed in 150 mM NETN lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin) for 30 min on ice. Cell extracts were clarified by centrifugation. For immunoprecipitation assays, cell lysates were incubated with c-Myc antibody tagged agarose beads (Sigma) at 4°C for 2 h. Beads were subsequently washed and boiled in SDS loading buffer. Proteins were separated using SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked in 5% milk phosphate-buffered saline/Tween and incubated with primary Ab against the Myc epitope (9e10, Santa Cruz, 1:500) or BLM (Abcam, 1:1000) for 1 h. Membranes were washed, and incubated with horseradish peroxidase-linked secondary Abs (Santa Cruz, 1:5000), and detected by chemiluminescence (Amersham).

For western blot detection of other proteins, the following primary antibodies were used: WRN, mouse monoclonal against Glutathione-S-Transferase tagged WRN_{1072–1432}, Spring Valley Labs

(Sykesville, MD), 1:1000; BRCA1, mouse monoclonal, Santa Cruz Biotechnology, 1:1000; RPA70, mouse monoclonal, Calbiochem (San Diego, CA), 1:1000; TopoIIα, rabbit polyclonal, Corgen (Taipei, Taiwan), 1:1000; FEN-1, mouse monoclonal, Abcam (Cambridge, MA), 1:1000; FANCA (kindly provided by Dr Weidong Wang, NIA-NIH); MLH1, mouse monoclonal, BD Biosciences Pharmingen (Basel, Switzerland), 1:1000.

DNA substrates

The 22 and 31 bp duplex DNA substrates were constructed as described previously (Gupta *et al*, 2006, 2007). PAGE-purified oligonucleotides used for the preparation of DNA substrates were purchased from Loftstrand Labs (Gaithersburg, MD).

Radiometric strand displacement helicase assays

BLM and FANCI helicase assay reaction mixtures (20 µl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 2% glycerol, 100 ng/µl BSA, 2 mM ATP, 10 fmol of the specified duplex DNA substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of FANCI or BLM helicases. WRN (Sharma *et al*, 2004) and RECQ1 (Sharma *et al*, 2005) reaction mixtures were prepared as described previously. Helicase reactions were initiated by the addition of FANCI, and were then incubated at 30°C for 15 min unless otherwise indicated. Reactions were quenched in the presence of a 10-fold excess of unlabelled oligonucleotide with the same sequence as the labelled strand to prevent reannealing, and the products were resolved on non-denaturing 12% (19:1 acrylamide:bisacrylamide) polyacrylamide gels. Unwinding was quantified as described previously (Gupta *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: ANS designed and performed the experiments and wrote the paper; NAR, YW, JAS, SS, GM, and PSN performed the experiments; SBC contributed reagents; IDH provided suggestions and comments and assisted with writing; and RMB designed the experiments and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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