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Fanconi anemia and Bloom's syndrome crosstalk through FANCI-BLM helicase interaction

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Abstract

Fanconi anemia (FA) and Bloom's syndrome (BS) are rare hereditary chromosomal instability disorders. FA displays bone marrow failure, acute myeloid leukemia, and head and neck cancers, whereas BS is characterized by growth retardation, immunodeficiency, and a wide spectrum of cancers. The *BLM* gene mutated in BS encodes a DNA helicase that functions in a protein complex to suppress sister chromatid exchange. Of the fifteen FA genetic complementation groups implicated in interstrand cross-link repair, *FANCI* encodes a DNA helicase involved in recombinational repair and replication stress response. Based on evidence that BLM and FANCI interact, we put forward that crosstalk between BLM and FA pathways is more complex than previously thought. We propose testable models for how FANCI and BLM coordinate to help cells deal with stalled replication forks or double strand breaks. Understanding how BLM and FANCI cooperate will help to elucidate an important pathway to maintain genomic stability.

Chromosomal instability disorders Fanconi anemia and Bloom's syndrome

A growing number of chromosomal instability disorders are linked to mutations in helicase genes that encode DNA unwinding enzymes implicated in cellular DNA replication, repair and recombination. Given that certain helicase diseases share similar mutant cellular phenotypes, it is probable that these helicases have common or partially overlapping functions to preserve genomic stability. We will focus on this hypothesis, placing particular emphasis on the crosstalk between FANCI and BLM helicases mutated in Fanconi anemia (FA) and Bloom's syndrome (BS), respectively.

FA [1] and BS [2] are characterized by growth retardation, reduced fertility, chromosomal instability, and predisposition to cancer. Distinguishing features of FA patients include congenital abnormalities, progressive bone marrow failure, acute myeloid leukemia, and head and neck cancers. BS patients display sunlight sensitivity, immunodeficiency, and a broad spectrum of cancers that appear early in life. Mutation of the *BLM* gene which encodes a DNA helicase is responsible for BS [3], and recent work using a mouse embryonic stem cell model supports a model that BLM plays early and late roles in homologous recombinational (HR) repair of double strand breaks (DSBs) [4]. The BLM-Topoisomerase III α (TopoIII α) protein complex (**Table 1**) can perform double Holliday junction (HJ) dissolution [5]. BLM is also implicated in DSB resection with other factors [6].

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FA mutant cells are highly sensitive to interstrand cross-linking (ICL) agents [7], and display elevated cross-linker induced sister chromatid exchange (SCE) [8]. Of the fifteen FA genes/candidate genes currently identified [9] (**Table 1**), two (*FANCM*, *FANCI*) encode DNA-dependent ATPases. *FANCM* regulates replication fork progression [10,11] and is thought to remodel stalled replication forks through its ATP-dependent branch migration activity [12-15]. *FANCM* and members of the FA core complex are required for efficient *FANCD2*/I mono-ubiquitination, a key activation step required for replication-coupled ICL repair [16].

FANCI (*BACH1*) helicase was originally discovered by its interaction with *BRCA1*, a tumor suppressor molecule implicated in DSB repair [17]. *FANCI*-deficient cells have a defect in DSB-induced HR and show delayed resolution of DSBs following ionizing radiation [18]. *FANCI* and certain other HR proteins function downstream of *FANCD2*/I mono-ubiquitination in ICL repair; however, their functions in ICL repair remain to be fully characterized.

BS cells exhibit elevated SCE [2]. Although not as dramatic as BS, increased SCE has been detected in chicken knockout cell lines (*FANCC* [19,20], *BRIP1* (*FANCI*) [21], and *FANCM* [22]), and human cells deficient for *FANCI* [23] or *FANCM* [24]. Human *FANCI* depletion resulted in elevated mitomycin C (MMC)-induced chromosomal abnormalities, including radial structures [18,21], similar to those observed in BS [25]. Like FA, BS cells are sensitive to compounds that induce ICLs [26]. The FA pathway and BLM collaborate during S-phase to prevent and during mitosis to resolve sister chromatid bridging at fragile sites that potentially leads to micronuclei and aneuploidy [27,28].

FA core complex and BLM directly interact to preserve genomic stability [29]. *FANCM* was shown to serve as a bridge connecting the FA core and BLM- TopoIII α complex [24]. The *FANCI*-BLM interaction is important for BLM stability and a normal response to the replication inhibitor hydroxyurea (HU) [23], leading us to propose that cross-talk between BLM and FA pathways is even more sophisticated than previously thought. Here, we define how *FANCI* and BLM helicases cooperate with each other, and propose testable models for how they collaborate to restart a stalled replication fork or facilitate DSB strand resection. Future research should address how *FANCI* regulates BLM protein stability and function, and understanding molecular and cellular defects of clinically relevant helicase mutations.

FA-BLM protein complexes

BLM was first identified in a *BRCA1* genome surveillance complex (BASC) with other DNA repair proteins (*MSH2*, *MSH6*, *MLH1*, replication protein A (RPA), ataxia telangiectasia mutated (*ATM*), *MRE11*-*RAD50*-*NBS1* (*MRN*)) [30]. BLM was also found to exist in a protein complex (BRAFT) that contained the FA core complex, RPA, and TopoIII α [29]. *FANCM* was shown to serve as an anchor protein to dock BLM-TopoIII α and FA core complexes at a DNA cross-link [24]. *FANCM* interaction with the FA core and BLM-TopoIII α complexes is required for SCE suppression and resistance to ICL agents, respectively.

Multiple BLM protein sub-complexes exist which may be regulated by cell cycle stage or cellular environment (*e.g.*, replicative stress). The BLM-*FANCI* interaction was enriched two-fold after cellular exposure to HU or MMC [23]. Moreover, BLM and *FANCI* foci partially co-localized in HU-treated cells. BLM and *FANCD2* also co-localize in MMC- and HU-induced foci and co-immunoprecipitate together [26]. A biochemical interaction between *FANCD2*, which binds DNA [31,32], and BLM (or *FANCI*) has not been reported; however, an intact FA pathway necessary for *FANCD2* mono-ubiquitination is required for BLM association with anaphase bridges [28].

BLM [33] and FANCI [34] interact with RPA. Demonstration that FANCI interacts with topoisomerase II β -binding protein 1 (TopBP1) to facilitate RPA loading onto chromatin and replication checkpoint activation [35] suggests FANCI participates with BLM in checkpoint signaling. Interaction of FANCI with mismatch repair complex MutL α is also required for a normal ICL-induced response [36]. It will be timely to determine if BLM (and FANCD2) play a role in the MutL α or TopBP1 signaling pathways with FANCI.

What are the biologically relevant situations and DNA substrates that engage BLM and its protein partner FANCI?

BLM and FANCI helicases together synergistically unwind a DNA substrate with discontinuity in the sugar phosphate backbone in the FANCI translocating strand [23]. The BLM-FANCI physical association may enable the dual helicase motor to act more processively in its unwinding function, particularly on damaged DNA or specific DNA structures. Coordinate action of a 3' to 5' DNA helicase (BLM) with a 5' to 3' helicase (FANCI) or possibly DNA translocase (FANCM) would provide a unique mechanism for unwinding or branch-migrating structured nucleic acids. It is not unprecedented that dual helicase motors operate in a synchronized manner [37]. We will discuss potentially relevant biological situations that invoke collaboration between FANCI and BLM.

Replication stress

Perturbation of normal replication fork progression poses a source of genomic instability. FANCI-deficient (EUFA30-F) cells like BS (PSNG13) cells are HU-sensitive [23]. Expression of a BLM-interacting FANCI fragment exerted a dominant negative effect on HU resistance by interfering with the FANCI-BLM association, suggesting that communication between BLM and FANCI helps cells deal with stalled forks.

To help cells cope with replication stress, BLM helicase can regress stalled replication forks to allow repair of the replication blocking lesion [38,39]. During fork regression, newly synthesized leading and lagging strands are unwound and annealed to one another to form a chicken-foot HJ-type structure. The regressed fork can then be metabolized by non-recombinogenic or recombinogenic pathways to enable fork resumption. We propose that FANCI may assist BLM in fork regression to enable base-pairing between unwound strands (**Fig. 1A**). Alternatively (or in addition), FANCI and BLM may together branch-migrate the regressed fork structure forward again so replication may resume. This would allow replication to restart in a non-recombinogenic manner, and prevent crossovers.

If the reversed fork HJ-like structure is cleaved by a structure-specific nuclease (*e.g.*, MUS81) to generate a DSB, then HR is elicited to repair the broken fork. BLM interacts with MUS81 and stimulates its endonuclease activity [40]; therefore, it may be that FANCI also interacts with MUS81 or other structure-specific nucleases to help repair forks. SLX4, a docking protein for the structure-specific nucleases MUS81-EME1, XPF-ERCC1, and SLX1, is also involved in cross-link repair in a manner dependent on FANCD2 monoubiquitination [41]. Defective HJ resolution leads to aberrant chromosome morphology in human cells [42], which may be suppressed by coordinate action of BLM and FANCI. Careful study of FA protein interactions should begin to elucidate mechanics of ICL repair and response to stalled forks.

Double strand break repair

In replicating cells, the highly toxic DSBs can be repaired by HR. A key intermediate in HR is the three-stranded D-loop that arises by RAD51-mediated invasion of a single-stranded DNA molecule into recipient duplex. BLM [43] or FANCI [44] may act to disrupt the D-

loop to inhibit inadvertent HR or regulate the process at an early stage between homeologous sequences. D-loop disruption by FANCI or BLM may also be relevant to synthesis-dependent strand annealing (SDSA) which follows a cycle of DNA duplex unwinding and strand annealing events to enable DSB repair. Genetic evidence has implicated *Drosophila melanogaster* BLM in SDSA [45], suggesting that mammalian BLM is also an active player. However, it is unclear if FANCI coordinates with BLM during SDSA.

BLM is implicated in an early stage of DSB repair in which strand resection from a DNA end occurs to provide a 3' single-stranded DNA tail for strand invasion [6,46,47]. Strand resection appears to be a complex process involving distinct constellations of helicases, nucleases, DNA binding proteins, and accessory factors. Concerted action of FANCI with BLM at an early step of DSB processing would be consistent with evidence that FANCI helicase participates in DSB repair [17,18]. We predict that strand resection is facilitated by FANCI and BLM helicases translocating on opposite strands of the broken double-stranded DNA end to promote access to structure-specific nucleases (EXO-1, DNA2) (**Fig. 1B**). The FANCI-BLM partnership may facilitate resection at chemically modified blocked DNA ends or through G-rich sequences prone to form G-quadruplex (G4) structures (next section). Careful examination of helicase and nuclease recruitment kinetics to localized damage in living cells should provide insight. Moreover, mammalian genetic systems should further help to decipher the mechanisms of DSB and ICL repair.

G-quadruplex DNA metabolism

G4 DNA consisting of stacked planar quartets composed of four guanines that interact by Hoogsteen hydrogen bonding can be formed by G-rich sequences widely distributed throughout the genome [48]. G4 structures potentially impede cellular DNA replication or transcription. Both FANCI [49,50] and BLM [51] were shown to efficiently unwind G4 DNA substrates, but require single-stranded tails of opposite polarities. A FA-J patient cell line (EUFA0030) was found to accumulate large genomic deletions in the locality of G4-forming sequences [49]. It remains to be seen if BLM-deficient cells also display genomic instability at regions characterized by the G4 DNA signature.

FANCI and BLM helicases may collaboratively act upon G-quadruplexes found in specific chromosomal regions under certain cellular (environmental) or genetic conditions. Specialized function of BLM and FANCI at telomeres is supported by the observation that they were found to be specifically bound to telomeric DNA in telomerase negative cells engaged in alternative lengthening of telomeres (ALT) maintenance pathway [52]. BLM is required for suppression of telomere fragility [53], and FANCI-deficient cells, but not FA-A nor FA-D2 cell lines, are hypersensitive to the G4-binding ligand telomestatin (TMS) [50], a compound that induces telomere capping defects [54,55]. The 3' to 5' BLM directionality seems better suited to enable a polymerase catalyze DNA synthesis through a G-rich sequence and avoid collision with replication machinery. However, biochemical results suggest that a G4 structure at the very 3' telomeric end would be resistant to BLM helicase and require other resolving mechanisms [56].

G-quadruplexes assume a number of different conformations in which one or multiple strands can be aligned in parallel or anti-parallel fashion with intervening loops [57]. FANCI and BLM may act upon intramolecular G-quadruplexes formed by human telomeric repeats in coordinate fashion to prevent capping defects. Depending on G-tetrad number and exposure of adjacent single-stranded regions, FANCI and BLM may initiate unwinding from internal or proximal regions of telomeric sequences, or intervening loops. Given evidence that translesion synthesis DNA polymerases η and κ are required for maintenance of guanine-rich DNA *in vivo* [58], future studies should determine if FANCI/

BLM facilitate translesion polymerase replication through G-rich elements. A relationship between FANCD1 and polymerase η has already been established, whereby FANCD1 uncoupled from BRCA1 promotes polymerase η -dependent bypass for cellular resistance to DNA cross-linking agents or ultraviolet light [59].

BLM protein stability is strongly dependent on FANCD1

BLM is degraded by a proteasome-mediated pathway in FANCD1-deficient cells in a specific manner since levels of BLM-interacting proteins (RPA, TopoIII α , FEN-1) were not affected [23]. Physical interaction between BLM and FANCD1 helps to stabilize BLM protein *in vivo*; however, the converse is not true, *i.e.*, FANCD1 level remained the same in BLM mutant and corrected cells. BLM levels are known to be affected when certain interacting partners (*e.g.*, RMI1 [60] and RMI2 [61]) are deficient, suggesting that BLM folding or susceptibility to proteasome degradation is highly influenced by interacting/associated proteins in FA or related pathways.

BLM sumoylation, DNA repair, and a potential FA connection

Demonstration that BLM is susceptible to proteasome-mediated degradation when its protein partner FANCD1 is absent suggests involvement of post-translational modification(s) in BLM protein stability. BLM is modified *in vivo* by the small ubiquitin-related modifiers (SUMOs) SUMO-2 and SUMO-3 [62]. This may be relevant to BLM stability since sumoylated residues can be bound by ubiquitin ligases that target proteins for ubiquitylation, which can lead to protein degradation through a proteasome pathway [63].

BLM sumoylation was already shown to have important biological consequences. BLM SUMO mutants failed to localize to promyelocytic bodies where BLM is thought to enact its DNA repair functions, and the cells accumulate more spontaneous [64] or HU-induced [65] γ H2AX foci than controls, attesting to the importance of BLM sumoylation for DNA damage accumulation. One would expect that replication fork damage would induce HR; however, this is not the case in SUMO-mutant BLM cells [65]. Moreover, RAD51 poorly localizes to damaged forks in SUMO-mutant BLM cells, which probably contributes to HR deficiency and DSB accumulation after replication stalling. Consistent with these data, sumoylated recombinant BLM binds RAD51 more efficiently than unsumoylated protein.

These observations raise the question if BLM sumoylation plays a role in the fate of BLM stability. If the absence of FANCD1 unmasks a SUMO site in BLM that is recognized by a ubiquitin ligase, then BLM may become destined for proteasome-mediated degradation. It was proposed that BLM sumoylation could promote HR-repair of broken replication forks through recruitment or retention of RAD51 at damaged forks [65]; however, in the absence of FANCD1, this critical role of BLM may be abolished since the helicase is degraded and/or the helicases fail to coordinately interact.

Future work should address the underlying mechanism whereby FANCD1 controls BLM stability and if they collaborate to repair broken replication forks in a RAD51-dependent manner. Signaling through a checkpoint kinase may affect BLM degradation when FANCD1 is deficient. This was observed for FANCD1 in which Chk1 prevents FANCD1 from being degraded by the proteasome after DNA damage [10]. The FA network is one in which protein partnerships exist to maintain stability of interacting proteins as well as coordinate functions to enact DNA repair processes. For example, MRN regulates FANCD2 stability and function during DSB repair [32].

FANCF clinical mutations and disease phenotypes

FA clinical symptoms may be partly attributable to the nature of the mutation. For example, a patient derived *FANCF-A349P* mutation in the iron-sulfur domain uncoupled ATPase and translocase activity from helicase activity or disruption of protein-DNA complexes [66]. Furthermore, *FANCF-A349P* expressed in normal cells impaired their resistance to DNA cross-linking agents or TMS. Clinical significance of the *FANCF-A349P* allele or other helicase-inactivating mutations in heterozygote carriers remains to be determined. Given evidence from cell-based models that dysfunctional helicase proteins exert a spectrum of dominant negative effects [67], future studies should directly evaluate genotype-phenotype relationships.

In some instances, a *FANCF* mutation may exert an effect on stability of BLM or other protein partners. Full-length *FANCF* protein was absent in certain FA-J patient cell lines, but residual full-length *FANCF* protein was detected in a cell line (EUFA776) harboring a *FANCF* missense mutation [68]. Reduced *FANCF* expression was reported for several breast cancer associated *FANCF* mutants [69]. Decreased BLM protein associated with *FANCF* deficiency may contribute to the nature and degree of chromosomal instability and associated phenotypes observed in FA-J patients or possibly individuals with a *FANCF* mutation predisposing them to breast cancer. Individuals with *FANCF* haploinsufficiency may display compromised DNA repair capacity due to reduced BLM. This hypothesis should be readily testable, and may also be relevant to cross-talk between *FANCD2* and *MRN*. Future studies of patient derived mutations will be informative for understanding the molecular basis of disease.

Concluding remarks and future perspectives

Future research should address if *FANCF*-BLM coordination is required for efficient restart of a stalled replication fork or DSB repair. Elaborate mechanisms that help cells deal with diverse forms of replication stress are likely to require catalytic action of *FANCF* and BLM helicases to enable smooth progression or fork restart. The next challenge is to define if specialized regions of the genome, such as telomeres or fragile sites, are targeted by the helicase partnership. Chromatin immunoprecipitation studies using cells exposed to agents that induce replication fork stalling may help to identify sequences enriched for interaction with both *FANCF* and BLM.

In terms of DSB repair, a useful approach would be to reconstitute DNA end resection with *FANCF* and its interacting proteins (BLM, *BRCA1*, RPA, and *MRN* (our unpublished data)) along with structure-specific nucleases (*DNA2*, *EXO-1*). These efforts will be significantly enhanced if chromatinized templates are used to better reflect the *in vivo* situation. Cell-based studies with defined substrates should provide a better understanding of *FANCF*'s role in DNA end resection and how its interaction with BLM is important. Genetic and cell biological experiments will be required to confirm and extend observations from biochemical studies.

The helicase interaction network is becoming increasingly more complex. Characterization of how BLM/*FANCF* post-translational modifications regulate their interaction is therefore a high priority. *FANCF* phosphorylation state was already shown to regulate its interaction with *BRCA1* [70]. Additional post-translational modifications of *FANCF* or BLM, such as sumoylation, are likely to be important in the regulation of pathway function.

There is still much to learn about the FA pathway and how it coordinates with other proteins to maintain genomic stability. Predisposition to cancer in BS and FA, as well as the connection of *BRCA*-associated FA proteins with breast cancer, suggest that BLM and

FANCF are indeed genome caretakers with tumor suppressor functions. A systematic investigation of patient mutations and FANCF-BLM interactions should yield new insights that will hopefully have translational applicability.

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Glossary

Anaphase bridges	a continuous string of chromatin stretching from one anaphase pole to the other. Anaphase bridges can generate micro-nuclei and lagging chromatin as a consequence of bridge rupture by the force of the mitotic spindle or during cytokinesis. Increased number of anaphase bridges and micro-nuclei are seen in Bloom's syndrome cells
Chicken foot	a four-stranded Holliday junction-like structure that is generated by annealing of leading and lagging strands and resumption of leading strand synthesis using the lagging strand as template
D-loop	a DNA structure (displacement loop) in which two strands of a double-stranded DNA molecule are separated for a stretch and held together by a third strand which contains base sequence that is complementary to one of the main strands with which it is paired
Helicase	an enzyme that catalytically unwinds structured nucleic acids by disrupting complementary hydrogen bonds in a reaction driven by the hydrolysis of nucleoside triphosphate
Holliday junction (HJ)	mobile junction between four strands of DNA that represents an intermediate in genetic recombination and important for genomic stability
Hydroxyurea (HU)	an antineoplastic drug compound that reduces production of deoxyribonucleotides by inhibiting the enzyme ribonucleotide reductase. Cellular exposure to HU induces replication stress
Interstrand cross-link (ICL)	a covalent linkage between complementary strands of a DNA double helix. DNA replication is blocked by cross-links, which cause replication arrest and cell death if the cross-link is not repaired
Mitomycin C (MMC)	a naturally occurring compound used as a chemotherapeutic agent that covalently introduces DNA cross-links. MMC is used in diagnostic tests for Fanconi Anemia since cells from these patients have genetic defects in DNA cross-link repair
Strand resection	process whereby a 5' end of a double strand break is trimmed back to create a 3' single strand overhang. Production of a 3' single-strand overhang permits homologous recombinational repair of the double strand break
Synthesis dependent strand annealing (SDSA)	a mechanism of double strand break repair during mitosis that allows for error-free repair of the break without exchange of adjacent sequences

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Figure 1. Potential roles of BLM and its interacting partner FANCI at stalled replication forks or double strand breaks

(a) Lesion on the leading strand template stalls replication (i). BLM/FANCI recruitment (ii) and fork regression facilitates template switching so that leading strand can be extended past the site of the lesion to create a chicken foot junction structure (iii). BLM/FANCI-mediated reversal of regressed fork permits restart of replication fork (iv). In the absence of junction cutting, replication would restart without SCEs. Black lines represent parental DNA strands and blue lines nascent DNA strands. (b) MRN complex senses DSB (i) and initiates end resection along with CtIP (ii). A BLM protein complex that either operates in the DNA2 pathway (BLM-DNA2-RPA-MRN) or the EXO1 pathway (EXO1-BLM-RPA-MRN) assembles at the DNA end (iii), and performs long 5' resection by DNA2 endonuclease or EXO1 exonuclease, respectively (iv). FANCI may putatively function in long strand resection by interacting with BLM in either of the resection complexes.

Table 1

Functions of FA and BLM-TopoIII α proteins

Complex	Protein	Protein function	Protein-complex function
Core complex	FANCA FANCB FANCC FANCE FANCF FANCG FANCL FANCM	Required for core complex stability Necessary for FANCD2/I mono-ubiquitination	ICL repair
		Hydrolyzes ATP; catalyzes HJ branch migration & fork regression	
ID complex	FANCD2 FANCI	Binds DNA structures Helps to properly mono-ubiquitinate FANCD2	ICL repair
Downstream FA proteins	FANCI (BACH1) FANCD1 (BRCA2) FANCN (PALB2) FANCO (RAD51C) FANCP (SLX4)	Unwinds DNA with 5'-3' polarity; interacts with BRCA1 Binds ssDNA and dsDNA; stimulates RAD51 strand exchange Required for stability of FANCD1 Binds ssDNA and hydrolyzes ATP Serves as scaffold for structure specific nucleases	
Bloom's-TopoIII α complex	BLM TopoIII α RPA RMI1 RMI2	Unwinds DNA with 3'-5' polarity; catalyzes HJ branch migration & fork regression Catalyzes ATP-independent ssDNA breakage Binds ssDNA Binds ssDNA Required for stability of BLM-TopoIII α complex	Double HJ dissolution

ssDNA: single stranded DNA; dsDNA: double stranded DNA; HJ: Holliday junction; ICL: interstrand cross-link.