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FANCI Helicase Operates in the Fanconi Anemia DNA Repair Pathway and the Response to Replicational Stress

Yuliang Wu and Robert M. Brosh Jr.*

Laboratory of Molecular Gerontology, National Institute on Aging, NIH, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA

Abstract

Fanconi anemia (FA) is an autosomal recessive disorder characterized by multiple congenital anomalies, progressive bone marrow failure, and high cancer risk. Cells from FA patients exhibit spontaneous chromosomal instability and hypersensitivity to DNA interstrand cross-linking (ICL) agents. Although the precise mechanistic details of the FA/BRCA pathway of ICL-repair are not well understood, progress has been made in the identification of the FA proteins that are required for the pathway. Among the 13 FA complementation groups from which all the FA genes have been cloned, only a few of the FA proteins are predicted to have direct roles in DNA metabolism. One of the more recently identified FA proteins, shown to be responsible for complementation of the FA complementation group J, is the BRCA1 Associated C-terminal Helicase (BACH1, designated FANCI), originally identified as a protein associated with breast cancer. FANCI has been proposed to function downstream of FANCD2 monoubiquitination, a critical event in the FA pathway. Evidence supports a role for FANCI in a homologous recombination (HR) pathway of double strand break (DSB) repair. In this review, we will summarize the current knowledge in terms of FANCI functions through its enzymatic activities and protein interactions. The molecular roles of FANCI in DNA repair and the response to replicational stress will be discussed.

Keywords

FANCI; Fanconi anemia; breast cancer; cancer; DNA damage; DNA repair; helicase

THE FANCONI ANEMIA PATHWAY

Fanconi anemia (FA) is a rare hereditary disorder characterized by skeletal abnormalities, bone marrow failure, and an increased incidence of cancer [1–3]. The basic cellular abnormality in FA has been postulated to lie in the DNA repair mechanisms because cells from FA patients display chromosomal abnormalities, and are hypersensitive to DNA interstrand crosslinks (ICLs) agents such as mitomycin C (MMC) and cisplatin. FA is a genetically heterogeneous disease with 13 complementation groups currently recognized. It is possible that more FA-associated genes may be identified since some FA patients have not been assigned a complementation group.

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 [4–7] or DNA processing molecules [8–10] in a DNA-damage response pathway. The eight FA gene products (FANCA/B/C/E/F/G/L/M) assemble to form the nuclear FA core

*Please send correspondence to: Robert M. Brosh, Jr., Ph.D., Laboratory of Molecular Gerontology, NIA, NIH, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA Phone: 410-558-8578, FAX: 410-558-8157, BroshR@grc.nia.nih.gov.

complex, and co-immunoprecipitation studies revealed that any known mutation in components of the core complex found in FA patients appears to disrupt integrity of the complex [11–14]. Two new proteins, FAAP24 and FAAP100, were recently found in this core complex and are essential for the stability of this core complex [15,16]. Certain FA proteins have been identified to be the same as proteins in the BRCA pathway or physically associated with the BRCA pathway. For example, FANCD1 (mutated in FA-D1) is identical to the breast/ovarian cancer susceptibility factor BRCA2 [17], and the most recently identified FA gene, *FANCN*, encodes PALB2 (partner and localizer of BRCA2) [14,18], a crucial regulator of the BRCA2 protein [19]. Additionally, FANCI is identical to BACH1/BRIP1 [20–22], a DNA helicase that interacts directly with BRCA1 [8]. Accumulating evidence has suggested that the proteins in the FA pathway constitute a novel DNA-damage response network that involves breast cancer proteins BRCA1 and BRCA2, which is more generally referred to as the FA/BRCA pathway.

Monoubiquitination of FANCD2 and its paralog, FANCI, play critical roles in the FA/BRCA pathway. Mutational inactivation of any one of the FA core complex results in loss of FANCD2 monoubiquitination and as a result, the loss of function of the FA repair pathway. FANCD2 monoubiquitylation and its nuclear foci formation occur in response to DNA damaging agents and during S phase of the cell cycle even in the absence of exogenous DNA damage [23]. A DNA damage-activated signaling kinase, ATR (ATM and rad3 related), and a single-strand DNA binding protein heterotrimeric complex Replication Protein A (RPA) are required for DNA damage-inducible monoubiquitylation and foci formation of FANCD2, indicating an upstream role for these factors in the activation of the FA pathway [4,11,12]. The exact signal and activation cascade required for FANCD2 monoubiquitylation, however, remain elusive. The newly discovered FANCI protein was shown to be monoubiquitylated in response to DNA damage and replication, and its monoubiquitylation also depends on the other FA core complex proteins [24–26]. FANCD1, FANCI and FANCN are thought to act downstream or in a pathway parallel to that of the FA pathway because cells lacking these proteins have normal levels of monoubiquitylated FANCD2 in response to DNA damage.

Recent evidence suggests that in addition to FANCI's requirement for FANCD2 and FANCI monoubiquitination, ATP-dependent activities of FANCI are required for MMC resistance but not FANCD2/FANCI monoubiquitination [27], suggesting that FANCI and its associated FA core complex plays a role in downstream events of the DNA repair pathway. Consistent with a direct role of FANCI in DNA repair, the protein has intrinsic DNA translocase [10] and branch-point translocase activities [9,27].

The FA proteins interact with a cast of DNA repair factors, and it is becoming increasingly evident that these FA-interacting proteins have a role in genome stability and their interactions are critical for a proper DNA damage response. For example, the Bloom's syndrome helicase (BLM) is associated with the FA core complex [12] and BLM mutations lead to a disease characterized by elevated sister chromatid exchange and cancer [28]. The FA/BRCA pathway and its cross-talk with other DNA repair proteins has been extensively reviewed elsewhere [1–3,13,29–32]. In this review, we will summarize the current knowledge in terms of FANCI functions through its enzymatic activities and protein interactions. The molecular roles of FANCI in DNA repair and the response to replicational stress will be discussed in terms of potential protein partners that operate in the FA pathway of DNA ICL repair and those that serve to enable smooth progression of the replication fork.

IDENTIFICATION AND CLASSIFICATION OF FANCI

FANCI was found to have a role in DSB repair by its interaction with the tumor suppressor BRCA1 [8,22] and be genetically linked to FA [20–22]. We will summarize the identification

of FANCD1 as a tumor suppressor and the classification of FANCD1 in a unique family of DNA helicases that are important for genomic stability.

Discovery of FANCD1 as a Helicase Associated with Breast Cancer and Linked to Fanconi Anemia

FANCD1 was first discovered by Cantor et al. as a protein that binds to the breast cancer C-terminal (BRCT) repeats of BRCA1 [8]. Because a transcription factor was also named BACH1 [33], the BRCA1-interacting helicase was later called BRIP1 (BRCA1 interacting protein 1). BACH1/BRIP1 was later identified as the gene mutated in the J complementation group of FA by three independent groups [20–22]. Now, BACH1 (BRIP1) is widely referred to as FANCD1 because of its genetic linkage to FA-J complementation group. A genetic interaction between FANCD1 and BRCA1 in DSB repair was established [8], suggesting a tumor suppressor role of FANCD1. In support of a clinical importance of FANCD1 as a tumor suppressor, two females among a cohort of 65 women with early-onset breast cancer were identified carrying 2 independent germ line sequence changes (P47A or M299I) in the FANCD1 coding region and normal genotypes for BRCA1 and BRCA2 [8]. More recently, Seal et al. reported that truncating mutations in FANCD1 are low-penetrance breast cancer susceptibility alleles [34]. A very recent study provided evidence that transcription of FANCD1 was controlled by the E2F/retinoblastoma pathway through a conserved E2F-responsive site, and its elevated expression is detected in primary invasive breast carcinomas with unfavorable characteristics [35]. Since FANCD2 monoubiquitination is intact in FA-J cells, FANCD1 appears to act downstream in the FA pathway akin to BRCA2/FANCD1 or in a parallel pathway to FANCD2 monoubiquitination. However, a clear understanding of the molecular and cellular roles of FANCD1 in relation to the FA pathway is lacking.

Classification of FANCD1 in a Unique Family of DNA Helicases

FANCD1 protein is 1249 amino acids in length, with a predicted molecular mass of ~130 kDa. The N-terminal 888 amino acid sequence of FANCD1 shares homology with proteins that belong to a DEAH subfamily of superfamily 2 helicases [8]. The BRCA1 interaction domain resides in the C-terminal region of FANCD1 after the helicase core domain (Fig. (1)). In addition to a core helicase domain comprised of the seven conserved ATPase/helicase motifs, the N-terminal region of FANCD1 has a predicted nuclear localization sequence (NLS) (residues 159–174) and a conserved Fe-S cluster (residues 270–363) (Fig. (1) and Fig. (2)). Three recently solved crystal structures of a protein that is mutated in Xeroderma pigmentosum group D (XPD) and related to FANCD1 by sequence homology in the helicase core domain confirmed the existence of a novel Fe-S domain [36–38], first identified biochemically in 2006 by the White lab [39]. Structural and biochemical evidence indicate that the conserved Fe-S domain is essential for XPD helicase activity and suggests that it serves as a recognition domain for the ssDNA-dsDNA junction, acting as the wedge for separation of complementary strands [36–39]. Biochemical characterization of a FANCD1-M299I variant (Fig. (2)) suggests that the conserved Fe-S domain in FANCD1 plays a role in unwinding damaged DNA substrates [40].

The Fe-S domain contains four conserved cysteines found in a number of helicases sharing sequence homology in the helicase core domain (Fig. (2)). The presence of Fe-S clusters in helicases engaged in the DNA damage response is interesting because a number of proteins involved in damaged DNA recognition and repair such as base excision repair glycosylases also contain Fe-S domains [41–43]. Biochemical and structural evidence indicates that Fe-S clusters in DNA repair glycosylases are involved in DNA binding, which ultimately impacts base removal catalysis [44]. DNA substrate binding by a glycosylase can facilitate oxidation of the $[4\text{Fe-4S}]^{2+}$ cluster to the $[4\text{Fe-4S}]^{3+}$ form, which has been proposed to help in locating damaged bases [44]. One model suggests that DNA-mediated charge transfer between DNA repair enzymes provides a mechanism to facilitate scanning of the DNA for damage searching

[45]. Most recently, an Fe-S cluster domain was discovered in human DNA primase that contributes to enzyme activity, raising the possibility that the DNA replication machinery has redox-sensitive activities [46]. It is of immediate interest to ascertain if DNA helicases involved in repair or replicational stress like FANCD1 utilize their Fe-S clusters to scan for DNA damage or alternate DNA structures during an early step of DNA damage processing.

The interaction domain of FANCD1 with MutL α , discussed later, was also mapped to a portion of the N-terminal helicase domain (residues 128–158) [47] (Fig. (1)). In the C-terminal region of FANCD1 (residues 888–1063) is located the BRCA1-interacting domain that directly binds to the two BRCT repeats of BRCA1. Since FANCD1 has been shown to exist in protein complexes [47–49], its interaction with other DNA repair factors such as the single-stranded DNA binding protein replication protein A (RPA) [50] may be mediated by the helicase domain or flanking regions.

A close inspection of the FANCD1 helicase domain reveals that this region of the protein shares sequence homology with a number of putative or bonafide DNA helicases that are implicated in the maintenance of genomic stability in a variety of organisms (Table (1)). The bacterial DNA damage-inducible G (DinG) helicase is regulated by the SOS circuit, and it has been suggested that DinG serves a backup role in DNA repair or replication restart after DNA damage [51]. Mutations in FANCD1-related genes in yeast (Chl1) and human (ChlR1) result in sister chromatid cohesion defects [52–54]. Moreover, loss of ChlR1 in mouse causes placental malformation and embryonic lethality due to the accumulation of aneuploid cells characterized by cohesion defects [55]. Nematodes mutated in the FANCD1 homolog *dog-1* (deletions of guanine-rich DNA) show germline as well as somatic deletions in genes containing guanine-rich DNA, suggesting a possible role in the metabolism of G-quadruplex structures [56]. Recent genetic evidence indicate that DOG-1 is the *C. elegans* FANCD1 homologue based on the sensitivity of *dog-1* mutants to ICL agents, and its epistatic relationship to *fcd-1* (*C. elegans* FANCD2) [57]. A mouse gene sharing sequence homology in the helicase domain with *dog-1* and FANCD1 is designated *Rtel* (Regulator of telomere length). *Rtel* knockout mice die within the first two weeks, and embryonic stem cells from these knockout mice show telomere loss and display many chromosome breaks and fusions, suggesting a role of the FANCD1 homolog in the maintenance of telomere length [58]. FANCD1 also shares sequence homology with the human XPD and yeast RAD3 helicases [8], which are subunits of the transcription factor IIIH (TFIIH)/DNA repair complex required for nucleotide excision repair [59, 60]. Mutation in XPD results in the skin cancer-prone disease Xeroderma pigmentosum [61]. Clearly, the FANCD1-like family of DNA helicases plays critical roles in DNA repair and genome integrity.

Pathogenic Mutations and Polymorphic Variations of FANCD1

FANCD1 missense and truncating mutations resulting in FA are found in the helicase core domain (see LOVD FA Database (<http://www.rockefeller.edu/fanconi/mutate/>)). Truncating FANCD1 mutations that cause FA in biallelic carriers confer susceptibility to breast cancer in monoallelic carriers [34]. Clinically relevant mutations exist in the conserved Fe-S cluster of the FANCD1 helicase domain [8,21,39,62] (Fig. (2)). A number of naturally occurring polymorphisms in FANCD1 exist whose pathological significance is not yet known. Characterization of the biochemical and cellular defects of pathogenic FANCD1 mutations and analysis of FANCD1 variants not yet linked to a human disease or cancer should be of wide interest to the field. In the following section, we will summarize what is known about the catalytic properties and protein interactions of FANCD1 to envision how FANCD1 acts in the FA pathway and other pathways.

DNA UNWINDING PROPERTIES OF THE FANCI HELICASE

Biochemical studies have begun to address the molecular functions of FANCI helicase, including its DNA substrate specificity and tracking mechanism. These studies suggest that FANCI has certain unique properties that are distinct from helicases of other families, and these functions are likely to be important for its mechanism of action and roles in cellular DNA metabolism.

DNA Substrate Specificity of the FANCI Helicase

The first biochemical characterization of the FANCI helicase showed it is a DNA-dependent ATPase that catalytically unwinds M13 partial duplex DNA substrates characterized by a covalently closed ssDNA circle annealed to a shorter oligonucleotide (Table (2)) in an ATP-dependent manner, and unwinds duplex DNA with a 5' to 3' directionality on a linearized M13 directionality substrate [62]. To determine the minimal loading requirements for optimal unwinding, FANCI was tested on oligonucleotide-based substrates with increasing lengths of the 5' ssDNA tail [63] (Table (2)). These studies demonstrated that FANCI requires a 5' tail of ~15 nucleotide (nt) to catalyze appreciable unwinding and that increasing the 5' ssDNA tail up to 35 nt increases the efficiency of FANCI helicase activity. Further analysis revealed that the additional presence of a 3' ssDNA tail, creating a forked duplex substrate (Table (2)), confers a significantly greater ability for FANCI to both bind and unwind the DNA molecule compared to a simple 5' tailed substrate [63]. Although neither the assembly state nor tertiary structure of FANCI is known, it is possible that the 3' tail may stimulate FANCI unwinding by sterically determining whether one or two DNA strands pass through a central channel of FANCI, as proposed for DnaB [64]. In support of the notion that FANCI may exist as a multimer, it was observed by gel exclusion chromatography that recombinant wild-type FLAG-tagged FANCI (~130-kDa polypeptide) migrates as a ~500-kDa enzymatically active species [62]. Future studies will likely address the potential importance of FANCI subunit interactions for its catalytic activity as a helicase in DNA repair pathways.

The preference of FANCI to unwind a forked duplex substrate with both 3' and 5' ssDNA arms raised the possibility that FANCI, like certain RecQ helicases (e.g., Werner syndrome helicase or BLM [65]), might have a relaxed DNA substrate specificity and unwind a variety of DNA replication and repair intermediates. FANCI was able to efficiently unwind a 5' flap DNA substrate (Table (2)), a strand displacement intermediate that arises during lagging strand synthesis or during DNA repair synthesis in a process such as base excision repair. However, FANCI failed to unwind a 3' flap substrate that might arise due to equilibrating strands during strand displacement. FANCI also failed to unwind a synthetic replication fork (with duplex leading and lagging strand arms) that might arise when the replication fork is stalled (Table (2)). These studies demonstrated that FANCI requires a pre-existing ssDNA tail of defined 5' to 3' polarity to unwind the adjacent B-form duplex DNA [63].

Since FANCI is proposed to be involved in HR repair, its activity on key DNA intermediates of this pathway is relevant. An early intermediate of HR is the three-stranded D-loop which is produced by strand invasion into a recipient duplex (Table (2)). FANCI was able to unwind D-loop structures by catalytically releasing the invading strand of the HR intermediate [63]. The four-stranded Holliday Junction (HJ) is a mobile DNA structure that occurs as an intermediate in homologous and site-specific DNA recombination and/or replication restart. Although FANCI could bind model HJ structures, it failed to unwind a synthetic HJ. The specificity of FANCI helicase for certain HR substrate intermediates suggests that FANCI might operate on the D-loop structure which arises in an early stage of HR after strand invasion, but not in the branch migration or resolution of a HJ that might form later in the HR process. Furthermore, the 5' to 3' directionality of FANCI helicase activity and its inability to unwind HJ structures suggests that FANCI is likely to operate in a distinct manner from the RecQ

helicases, which are also implicated in HR repair. This does not exclude, however, the possibility that a RecQ helicase may co-localize and operate with FANCI in a DNA repair center or even a direct interaction between the two helicases.

FANCI helicase activity was found to be 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 [63]. Importantly, these studies revealed that FANCI does not display strand-specific inhibition with respect to the strand that it translocates on as observed for other helicases (vaccinia virus NPH-II [66], *E. coli* Dda [67,68], bacteriophage T7 gene 4 [69]). The ability of FANCI to sense backbone discontinuity in both strands of the DNA duplex as it unwinds may be important for its physiological functions in DNA damage signaling or repair. The existence of FANCI in a BRCA1-containing complex with other DNA repair factors involved in the recognition and repair of aberrant DNA structures suggests that the complex functions in the DNA damage response [49]. It is conceivable that a specialized helicase like FANCI might have the ability to sense DNA damage in either strand of the duplex and facilitate DNA damage processing.

In addition to DNA damage in standard duplex DNA, non-B form DNA with alternate secondary structure may impede the replication fork, and be a source of genomic instability (Fig. (3)). Guanine-rich nucleic acids have the potential to form G-quadruplex or 'G4 DNA' stabilized by Hoogsteen hydrogen bonding between guanine residues, and G4 DNA influences gene expression and genomic stability (for review, see [70]). The number of distinct sites with potential to form G4 DNA in the human genome is estimated at more than 300,000 [71]. The unique metabolism of G-rich chromosomal regions that form quadruplexes may influence a number of biological processes including immunoglobulin gene rearrangements [72], promoter activation [73], and telomere maintenance [74,75].

FANCI was shown to efficiently unwind a variety of model G4 substrates in a reaction that required the presence of ATP and was dependent on intrinsic FANCI ATP hydrolysis [76]. A 5' ssDNA tail is required for FANCI to load and unwind the adjacent G-tetraplex. The 5' tail requirement distinguishes FANCI from the human WRN or BLM syndrome helicases that require a 3' ssDNA tail to unwind G4 substrates [77,78]. Evidence that the FANCI helicase activity on G-quadruplex DNA is biologically important is discussed below in the *Replication* section.

DNA REPAIR PROTEINS TEAM UP WITH FANCI TO INSURE A PROPER DNA DAMAGE RESPONSE

Understanding the pathway(s) that FANCI participates in to facilitate an appropriate DNA damage response will require a thorough analysis of its protein interactions and genetic functions. We will summarize our current knowledge of how FANCI performs its cellular functions through its timely interactions with DNA repair factors.

Interaction of FANCI with the Tumor Suppressor BRCA1 Is Required for Normal Double Strand Break Repair

Germ line mutations in *BRCA1* lead to an increased lifetime risk of breast and/or ovarian cancer [79]. Moreover, the tumor suppressor gene *BRCA1* is required for the maintenance of chromosomal stability and DNA damage resistance (for review see [80–82]). The nuclear phosphoprotein BRCA1 contains tandem C-terminal BRCT motifs, a conserved protein sequence found in a large number of DNA damage-response proteins [83]. The integrity of the BRCT motifs is required for the role of BRCA1 in DSB repair [82] and HR [84–87]. Tumor-

predisposing missense and deletion mutations in the *BRCA1* BRCT domain, all of which render *BRCA1* defective in its DSB repair function, also disrupt the ability of *BRCA1* to bind *FANCI*. A role of *FANCI* helicase in DSB repair was suggested by the observation that overexpression of a *FANCI* allele (K52R) carrying a mutation in its ATP-binding pocket that inactivates its ATPase/helicase function [62] resulted in a marked decrease in the ability of cells to repair DSBs, and that this dominant negative phenotype depended on a specific interaction between *FANCI* and *BRCA1* [8]. These results suggested that the role of *FANCI* in DSB repair operates in a manner dependent on its association with *BRCA1*. Structural evidence demonstrated that two intact *BRCA1* BRCT repeats as well as phosphorylation of *FANCI* are critical for *BRCA1* binding to *FANCI* [88–90]. The *FANCI*-*BRCA1* interaction is cell cycle regulated and required for the G2/M checkpoint control in response to DNA damage [90,91]. The importance of *FANCI* helicase activity in tumor suppression was supported by the identification of two naturally occurring *FANCI* missense mutations genetically linked to cancer that impair the DNA unwinding activity of the enzyme [8,62]. Thus, the tumor suppressor role of *FANCI* likely involves both its helicase activity and interaction with *BRCA1*. The preferential binding of *BRCA1* to forked DNA structures [92] may help *FANCI* load on to a critical DNA repair intermediate such as D-loop structure that the enzyme can catalytically unwind.

BRCA1-FANCI Protein Complexes Exist in Cells

FANCI was initially identified through Far Western blotting using phosphorylated BRCT repeats of *BRCA1* [8]. It was subsequently reported that phosphoserine 990 of *FANCI* is critical for *FANCI* binding to BRCT motif of *BRCA1* [88–90]. Furthermore, phosphorylated serine at 990 of *FANCI* also associates with the BRCT motifs of Topoisomerase II β binding protein 1 (TopBP1) and *BRCA1*-associated RING domain protein (BARD1) [90,91]. So far, only a limited number of proteins are reported to associate with *FANCI*, as listed in Table (3).

An important area of study has been the formation of DNA damage-induced protein complexes that mediate the DNA damage response. Since *BRCA1* plays a key role in the constellation of protein complexes, a number of studies have focused on the assembly of DNA repair centers with *BRCA1* as their focal point. Greenberg and colleagues reported that *BRCA1* is required for the transport of *FANCI*, BARD1, *BRCA2*, and Rad51 to sites of DNA damage where other proteins such as the MRE11-RAD50-NBS1 complex associate [49]. The assembly of a *BRCA1*/*FANCI*/BARD1 complex enables the interaction of *BRCA1*/*FANCI* with TopBP1, a factor that plays an important role in the execution of the S phase checkpoint [49]. Modulation of DNA damage checkpoint function by this complex may contribute to tumor suppression.

FANCI protein complexes were identified with mismatch repair factors (discussed further below) and with *BRCA1*/*BRCA2*/BARD1, the latter of which shows an increased association with chromatin during S phase [48]. The *FANCI* complex is distinct from that of *BRCA1*-*BRCA2*-containing complex (BRCC), as it does not contain either BRCC36 (a 36 kDa protein in BRCC complex) or BRCC45/BRE (a 45 kDa protein in BRCC complex)[48]. Understanding the assembly, activities, and functions of *FANCI* protein complexes in DNA damage response pathways remains a formidable challenge and worthy endeavor.

RPA Interacts with FANCI in a DNA Damage-Inducible Manner and Stimulates its Helicase Activity

FANCI was observed to efficiently unwind a short duplex of 20 bp, but severely limited in unwinding a 47 bp substrate, suggesting that the enzyme is not very processive [50]. However, in the presence of the single-stranded DNA binding protein RPA, *FANCI* unwinds the longer duplex substrate much more efficiently. Stimulation of *FANCI* helicase activity by RPA is specific since heterologous single-stranded binding proteins do not increase *FANCI* helicase activity to any significant extent. The mechanism for RPA stimulation of *FANCI* helicase

activity may be more complex than simply a role of RPA to coat the unwound ssDNA tracts left behind the advancing helicase. Consistent with this idea, FANCD1 directly binds with high affinity to the RPA70 subunit of the heterotrimer. FANCD1 and RPA in human nuclear extracts were shown to coimmunoprecipitate with each other and colocalized after DNA damage induced by ionizing radiation (IR) or MMC or replicational stress induced by hydroxyurea [50]. Since FANCD1 is implicated in a downstream event of FANCD2 monoubiquitination, the possibility that a mutation in an upstream member (core complex protein FANCA) of the FA pathway might affect the interaction of FANCD1 and RPA was investigated. FANCD1 and RPA were co-immunoprecipitated with each other from extracts of FA-A cells, suggesting that the interaction is not dependent on upstream events of the FA pathway. Likewise, FANCD1 and RPA were coimmunoprecipitated with each other from extracts of FA-D2 mutant cells, suggesting that the FANCD1-RPA interaction can occur independently of the classic FA pathway altogether.

The Interaction of MutL α with FANCD1 Is Essential for Cross-Link Repair

Although the interaction of FANCD1 and BRCA1 was found to be important for DSB repair [8], Cantor and colleagues determined that FANCD1 binding to BRCA1 was not required to correct the ICL-induced cell cycle progression defect in FA-J cells [47]. Thus, FANCD1 operates independent of BRCA1 to correct FA-J cells, a result that supports the finding that FANCD1's role in the FA pathway is independent of BRCA1 in chicken cells [93]. In an effort to identify other FANCD1 protein interactions that might be important for the ICL response, Peng et al. used a two-step immunoaffinity strategy with human cells expressing a double-tagged FANCD1 construct to identify novel protein partners [47]. FANCD1 was found to exist in a protein complex with the mismatch repair proteins MLH1-PMS2 (MutL α). Like FANCD1, MutL α was found to function downstream of FANCD2 monoubiquitination. FANCD1 was shown to directly interact with MLH1 independent of BRCA1, and this DNA-independent interaction was mapped to a defined region (residues 128–158) within the FANCD1 helicase domain. Disruption of the native MLH1/FANCD1 interaction by expression of an eGFP-tagged FANCD1_{128–158} fusion protein resulted in poorer cell survival after MMC treatment compared to expression of eGFP. Further mapping revealed that FANCD1 residues Lys 141 and Lys142 were critically important for MLH1 binding, but not FANCD1 helicase activity, and were essential to correct the sensitivity of FA-J cells to agents that induce ICL and consequently interfere with cell cycle progression, resulting in 4N DNA accumulation [47]. In sum, genetic studies demonstrated that FANCD1 helicase activity and MLH1 binding, but not BRCA1 binding, is required to correct the cross-link response in FA-J cells. Although mismatch repair proteins have been implicated in the sensing and/or processing of ICLs, this study provided the first evidence for a direct link between FA and mismatch repair, leading to the prediction that FANCD1 has a broader role in DNA damage signaling independent of BRCA1 [47].

It was speculated that the MutL α -associated mismatch repair complex serves to mobilize or regulate FANCD1 DNA unwinding at the site of DNA damage, which facilitates the repair of the lesion. Alternatively, FANCD1 may catalyze protein displacement and remove the mismatch repair complex from DNA, enabling the repair machinery access to the damaged DNA and also alleviate prolonged G2/M arrest.

How Might FANCD1 Helicase Operate in ICL Through HR Repair?

The seminal demonstration that FANCD1 interacts with BRCA1 and that cells harboring a FANCD1 mutant defective in its interaction with BRCA1 fail to repair DSBs suggests that the helicase functions in DSB repair by promoting HR [8]. FA-J cells, like other FA mutant cells, are hypersensitive to agents that induce DNA ICLs. Of the thirteen gene products implicated in the FA pathway, only FANCD1, FANCD1 (BRCA2) and the binding partner of FANCD1 (FANCD1) operate downstream of FANCD2 monoubiquitination. It was proposed that FANCD1

may operate with BRCA2 during an early step of HR repair to set up proper Rad51 protein filament formation on ssDNA as an early step in HR repair. Consistent with this possibility, FANCI interacts with RPA, a protein that assembles on processed ssDNA ends at sites of DSBs prior to Rad51 filament formation. A recent review that focuses on the mechanisms of HR in DSB and ICL repair and DNA damage tolerance suggests a potential role for a 5' to 3' helicase in gap repair to resolve lagging strand blockage [94]. In this model, Li and Heyer proposed that recombinational repair of the gap on the lagging strand involves dislodging of the blocked strand by a 5' to 3' helicase, assembly of Rad51 on the ssDNA, and formation of the D-loop. However, contradictory to the model that FANCI operates upstream of Rad51 is the observation that Rad51 focus formation after hydroxyurea (HU) exposure is not dependent on FANCI [22]. It is conceivable that a predicted early step of FANCI in HR repair is restricted to a specific pathway(s) such as that elicited by a replication fork encounter with an ICL. Other possible roles for FANCI in the metabolism of ICLs exist such as the facilitation of translesion synthesis by promoting loading of a translesion polymerase through unwinding of DNA structures associated with stalled replication forks or dissociation of protein-DNA complexes [2].

A compelling model was proposed to explain the biological significance of the BRCA1-FANCI interaction and to reconcile the observations that Rad51 foci are formed in FANCI-deficient cells [22] whereas BRCA1 is required for subnuclear assembly of Rad51 foci after DNA damage [95,96]. Cantor and Andreassen suggested that FANCI functions to disrupt Rad51 protein-DNA filaments to complete HR, and BRCA1 regulates FANCI by preventing the motor ATPase from prematurely removing Rad51 polymer from ssDNA involved in HR, which in turn would reduce chromosomal instability [97]. According to the authors, this model predicts that inhibition of FANCI in BRCA1-deficient cells may lead to increased cell survival after DNA damage. This possibility should be readily testable, and if true, would suggest that targeting FANCI as a mechanism to enhance DNA damaging anti-cancer therapy in BRCA1-deficient cells is potentially problematic.

A ROLE OF FANCI IN DNA REPLICATION

There has been some discussion that in addition to a role in ICL repair, some FA proteins may function to stabilize the replication fork during conditions of replicational stress [98,99]. A recent study using *Xenopus laevis* oocyte extracts shows that FANCL is required to stabilize the replication fork [100]. As a DNA helicase, FANCI is a likely candidate to operate in this capacity, potentially extending its range of function beyond cross-link repair. We will explore this idea further, presenting recent discoveries in support of a function for FANCI in DNA replication.

FANCI Is Required for Timely Progression Through S Phase

A recent paper by Kumaraswamy and Shiekhhattar experimentally addressed the hypothesis that FANCI plays a role during S phase [48]. They observed that acute depletion of FANCI by RNA interference resulted in an increased number of cells in the G1 phase of the cell cycle 6 hr after release from the DNA polymerase inhibitor aphidicolin. Furthermore, bromodeoxyuridine (BrdU) incorporation studies demonstrated reduced DNA synthesis in the FANCI-depleted cells after release from aphidicolin, suggesting that FANCI is necessary for timely progression through S phase. Consistent with this, FANCI was found to show enhanced association with chromatin and display elevated DNA-dependent ATPase activity, activated by FANCI dephosphorylation, as cells progressed through S phase [48].

To assess the role of the FANCI ATPase or its BRCA1-interacting domains, cells expressing a catalytically inactive FANCI ATPase mutant (K52R) or BRCA1-interactive defective mutant (S990A) were examined with the hypothesis that the FANCI domain mutants might exert a

dominant negative effect [48]. Cell lines expressing FANCD1 mutants defective in either ATP hydrolysis or interaction with BRCA1 displayed a defect in S phase progression, increased IR sensitivity, and activation of the DNA damage checkpoint 53BP1 and the presence of DSBs as demonstrated by elevated γ H2AX phosphorylation [48]. Altogether, these observations led the authors to propose that FANCD1 may have a role in resolving difficult structural motifs encountered by the replication forks during DNA replication. Defects in FANCD1 helicase activity would lead to stalled replication forks, which in turn impede S phase progression. The interrupted replication forks would ultimately give rise to DSBs and chromosomal instability.

FANCD1 Resolves G-Quadruplex DNA Structures that Impede Replication and Pose a Source of Genomic Instability

The requirement for activation of FANCD1 helicase activity to achieve timely progression through S phase of the cell cycle suggested a role of FANCD1 in some aspect of replication [48]; however, its precise functions remain to be understood. Failure to replicate DNA in a timely and faithful manner can result in mutations, genomic instability, and cellular dysfunction manifested by cell transformation, senescence, or death. To deal with replication blocking lesions, proteins associated with the DNA replication fork or involved in distinct DNA damage response pathways facilitate fork progression or the accurate repair and restart of damaged or broken replication forks.

It was proposed that the peculiar type of genomic instability in mutants of *C. elegans dog-1*, a FANCD1 homologue that also functions in ICL repair [101], was due to the accumulation of secondary DNA structures in G-rich tracts [56]. A recent study showed that sequences which match the G4 DNA signature are deleted in *dog-1* mutants [102]. Another study showed that there is not only PolyG/C tract deletions in *dog-1* mutants, but also large chromosomal rearrangements, including duplication, translocation and deficiencies [103]. However, demonstration that DOG-1 is a bonfide ATP-dependent helicase on G-quadruplexes or even duplex DNA substrates remains to be shown. DOG-1 may interact with accessory factors that modulates its activity. FANCD1 G4 DNA unwinding was found to be differentially modulated by DNA repair factors that directly interact with FANCD1 [76]. The single-stranded DNA binding protein RPA stimulated FANCD1 G4 unwinding in a specific manner, whereas the mismatch repair complex MSH2/6 (MutS α), which interacts with G4 DNA with high affinity [72], inhibited FANCD1 G4 helicase activity. The strong co-localization of FANCD1 and RPA in response to nucleotide depletion by hydroxyurea [50] suggests that the two proteins collaborate in situations of replicational stress, a condition that arises when the replication fork is impeded by a G4 structure.

FANCD1-depleted cells were found to be sensitive to telomestatin (TMS) which selectively interacts with G-quadruplex DNA [104]. FANCD1-depleted cells treated with TMS displayed impaired proliferation, elevated apoptosis and DNA damage compared to siRNA control cells [76], suggesting that G4 DNA represents a physiological substrate of FANCD1. Although the FA pathway has been classically described in terms of ICL repair, the cellular defects associated with *FANCD1* mutation may extend beyond the reduced ability to repair ICLs and involve other types of DNA structural roadblocks to replication (Fig. (3)).

SUMMARY AND PERSPECTIVE

An in-depth understanding of the molecular pathogenesis of FA and breast cancer is only beginning to emerge. As a tumor suppressor and genome caretaker, FANCD1 stands at the crossroads of these diseases. Understanding the role of FANCD1 in DNA repair and the maintenance of genomic stability is a formidable challenge, but one of great importance. Future work will likely lead to a more detailed comprehension of the role of FANCD1 in the classic FA pathway as well as its involvement in the response to replicational stress. Furthermore,

dissection of the molecular functions and interactions of FANCD1 in cellular DNA metabolism should lead to new insights for FANCD1 and FANCD1-like helicases in chromosomal integrity and disease-related pathways.

Abbreviations

ATM, ataxia-telangiectasia mutated
 ATR, ATM and rad3 related
 BARD1, BRCA1-associated RING domain protein
 BLM, Bloom's syndrome helicase
 BRCC, BRCA1-BRCA2-containing complex
 BRCC36, a 36 kDa protein in BRCC complex
 BRCC45/BRE, a 45 kDa protein in BRCC complex correspond to the brain- and reproductive organs-specific gene, BRCT: Breast cancer C-terminal BRE
 BrdU, bromodeoxyuridine
 DinG, damage-inducible G helicase
 DSB, double strand break
 dog-1, deletions of guanine-rich DNA
 FA, Fanconi anemia
 H2AX, Histone isoform 2AX
 HJ, Holliday Junction
 HR, homologous recombination
 HU, hydroxyurea
 ICLs, interstrand cross-links
 IR, ionizing radiation
 MMC, mitomycin C
 NLS, nuclear localization sequence
 nt, nucleotide
 NER, nucleotide excision repair
 RPA, Replication Protein A
 Rtel, Regulator of telomere length
 TFIIH, Transcription factor IIH
 TMS, Telomestatin
 TopBP1, Topoisomerase II β binding protein 1
 XPD, Xeroderma pigmentosum group D

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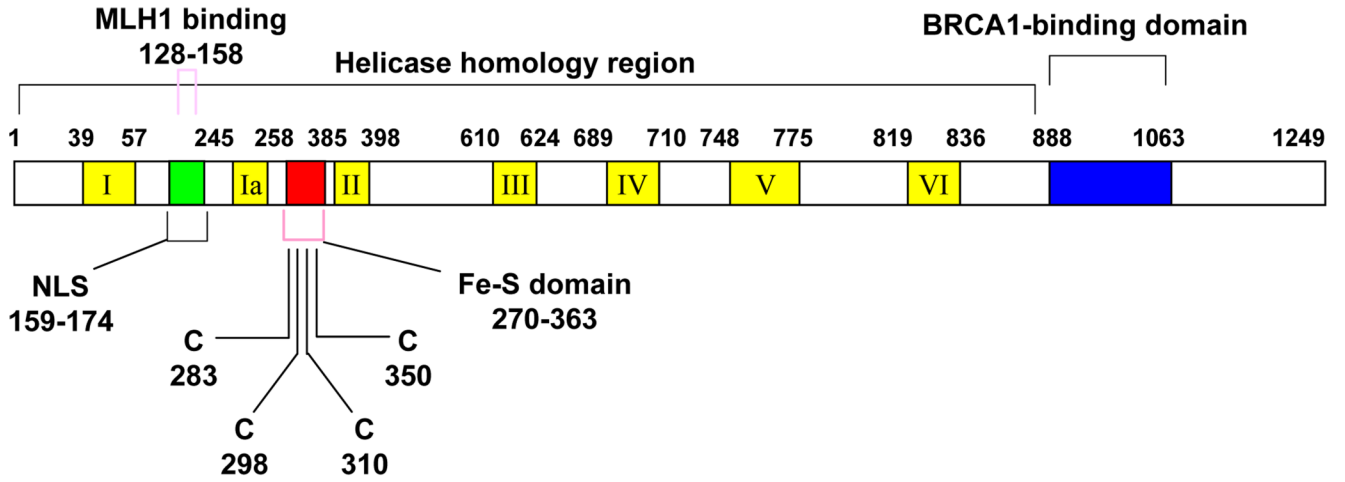


Fig. (1). Map of FANCD1 protein

The conserved helicase motifs are indicated by yellow boxes and the positions of the metal binding domain (red), nuclear localization sequence (NLS in green), and BRCA1 interaction domain (blue). MLH1 binding domain and four conserved cysteines in iron-sulfur domain are indicated above and below the protein schematic in pink.

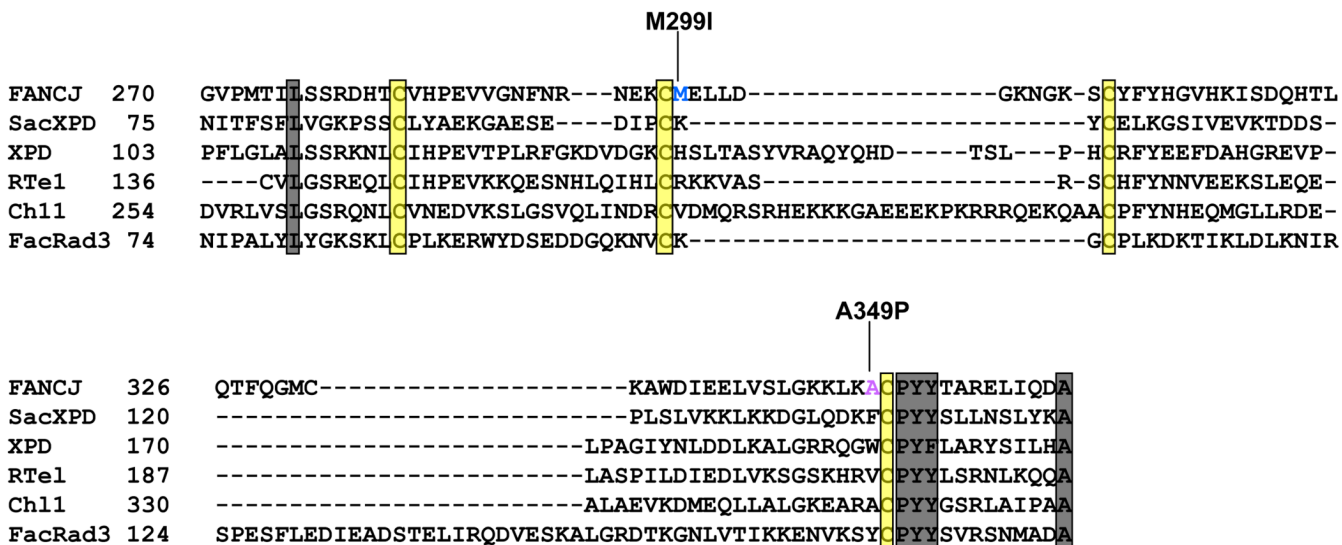


Fig. (2). Sequence alignment of the Fe-S domain

Human DNA helicases FANCJ and XPD, mouse RTel, yeast Ch11, and *Sulfolobus acidocaldarius* (Sac) XPD and *Ferroplasma acidarmanus* (Fac) Rad3 were shown. The four conserved cysteine residues of the metal binding domain are highlighted in yellow. The FANCJ amino acid substitution (M299I) adjacent to a conserved cysteine residue and representing a FANCJ polymorphic variant is highlighted. Another substitution (A349P) arising from a mutation genetically linked to Fanconi Anemia (Complementation Group J) and adjacent to a conserved cysteine residue is also highlighted. Some conserved residues (L, PYY and A) are highlighted in grey.

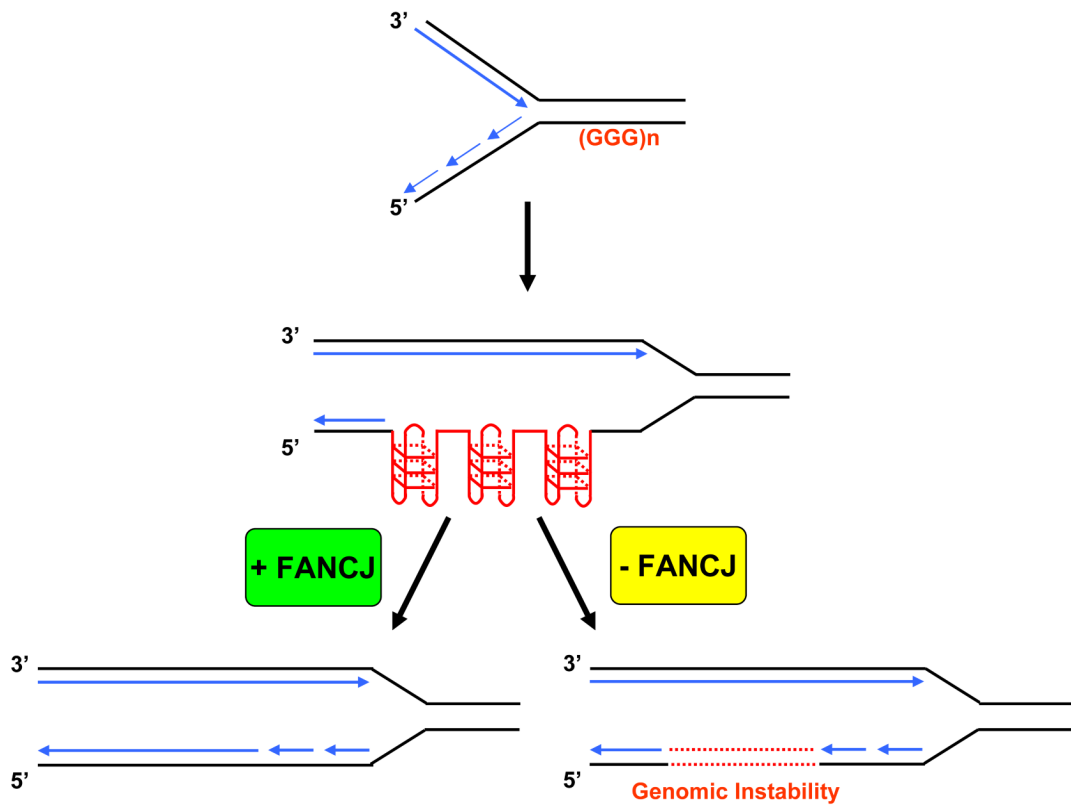


Fig. (3). Proposed role of FANCD1 in resolving G4 quadruplex DNA during replication

According to the model presented, the replication fork moves in from the left toward the poly (G) sequences (in red). Single-stranded poly(G) sequences used as a template for lagging-strand DNA synthesis are postulated to occasionally give rise to stable secondary structures such as G4 structure. FANCD1 helicase is recruited to unwind the G4 quadruplex, permitting polymerization using the template strand (in blue). Failure to resolve G-quadruplex DNA in FA-J cells results in genomic instability (dashed line in red).

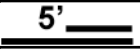


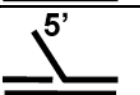





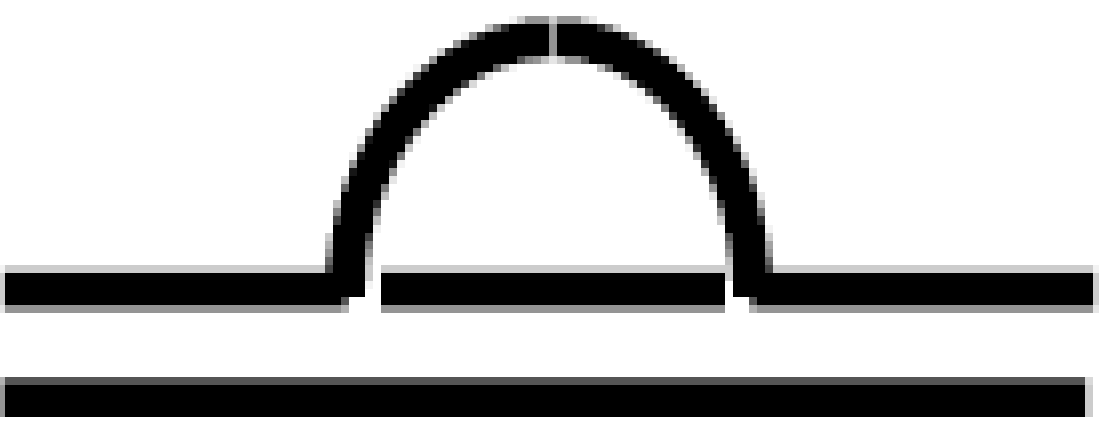
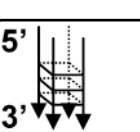
Table 1


FANCI-like DNA Helicases


Organism	Helicase	Disease and Organic or Cellular Phenotype	Proposed Function	References
Human	FANCI	Fanconi anemia and ICL sensitivity	ICL repair/HR repair, G4 resolution	[8,62,76]
	ChR1	Sister chromatid cohesion defects	Sister-chromatid cohesion	[52]
	XPD	Xeroderma pigmentosum and UV sensitivity	NER	[61]
Mouse	Rtel1	Telomere loss and chromosome instability	Telomere maintenance	[58]
	ChR1	Sister chromatid cohesion defects	Sister chromatid cohesion	[55]
Nematode	DOG-1	Deletion of G-rich DNA	G4 resolution, ICL repair	[56,57,101]
Yeast	Rad3	UV sensitivity	NER	[59]
	CHL1	Unusual mating and cell cycle delay at G2/M, sister chromatid cohesion defects	Sister chromatid cohesion	[54,105,106]
Bacteria	DinG		Recombinational repair	[51]

Table 2

Substrate Specificity of FANCI Helicase

Substrate Name	Substrate Structure	Relative Helicase Activity
3'-tailed duplex ^a		No
5'-tailed duplex ^a		+
Forked duplex ^a		+++
5' flap substrate ^a		+++
3' flap substrate ^a		No
Replication fork ^a		No
Holliday junction ^a		No
D-loop with a 5' ssDNA tail ^a		+++
D-loop with a 3' ssDNA tail ^a		++
Flush D-loop ^a		+
G-quadruplex DNA ^b		+++

Substrate Name	Substrate Structure	Relative Helicase Activity
M13 partial duplex DNA ^c	<p data-bbox="305 254 1430 415">19-mer DNA</p>  <p data-bbox="678 785 1089 953">M13</p>	++

Substrate Name	Substrate Structure	Relative Helicase Activity
M13 partial DNA-RNA hybrid ^c	<p style="text-align: center; font-size: 2em; font-weight: bold;">24-mer RNA</p> 	+
RNA double strand ^c	<p style="text-align: center;"> 5' RNA <hr style="width: 100%; border: 1px solid black;"/> RNA </p>	No

^a See text and ref 63

^b ref 76

^c ref 62 for details.

Table 3

Proteins that interact with FANCD1

FANCD1 Interacting Proteins	Physical Association	Functional/Genetic Interaction	DNA Damage Response	References
BRCA1	FANCD1 binds to the BRCT domain of BRCA1	Physical interaction required for DSB repair	DSB and HR repair	[8,48,88–91]
MLH1 - PMS2	MLH1 binds to helicase domain of FANCD1 (128–158 aa)	Physical interaction required for ICL repair	ICL repair	[47]
RPA	FANCD1 binds to RPA70 subunit	Stimulation of FANCD1 helicase activity	Co-localization after MMC, IR or HU	[50]
TOPBP1	FANCD1 associates with BRCA1	ATR activation	DNA damage signal transduction	[49,90]
BARD1	FANCD1 associates with BRCA1	Required for ubiquitin-ligase activity of BRCA1	DSB repair	[48,90]