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REVIEW

Fanconi Anemia: at the Crossroads of DNA Repair

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Abstract—Fanconi anemia (FA) is an autosomal disorder that causes genome instability. FA patients suffer developmental abnormalities, early-onset bone marrow failure, and a predisposition to cancer. The disease is manifested by defects in DNA repair, hypersensitivity to DNA crosslinking agents, and a high degree of chromosomal aberrations. The FA pathway comprises 13 disease-causing genes involved in maintaining genomic stability. The fast pace of study of the novel DNA damage network has led to the constant discovery of new FA-like genes involved in the pathway that when mutated lead to similar disorders. A majority of the FA proteins act as signal transducers and scaffolding proteins to employ other pathways to repair DNA. This review discusses what is known about the FA proteins and other recently linked FA-like proteins. The goal is to clarify how the proteins work together to carry out interstrand crosslink repair and homologous recombination-mediated repair of damaged DNA.

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A DNA double-strand break is the most toxic form of DNA damage that can occur in a proliferating cell [1]. If DNA repair does not occur, the cell's normal function will cease and it will undergo apoptosis. As DNA damage accumulates, proliferating cells are eliminated and tissue homeostasis is compromised. Alternatively, DNA double-strand breaks may lead to mutations. Increasing loss of tissue homeostasis and accumulation of mutations is a hallmark of cancer and accelerated aging [1-3]. To combat this problem, the cell employs DNA repair pathways including homologous recombination, nonhomologous end joining, nucleotide excision repair, translesion bypass, and the newly discovered Fanconi anemia (FA) pathway to maintain genomic integrity.

DNA repair proteins regulate crucial steps along these pathways by either playing a role in DNA damage signaling or by directly processing the damaged DNA through intrinsic catalytic activities [4]. Inactivating mutations of these DNA repair proteins are known to lead to development of cancer and premature aging disorders such as FA, Bloom's syndrome, and Werner's syndrome. Patients with these diseases have skin pigment alterations, decreased blood cell production, degenerative loss of skeletal muscle, osteoporosis, endocrine disorders, and other symptoms commonly associated with the normal process of aging [5, 6]. All of these symptoms stem from the inability of the body to recover fully after genotoxic stress and increased genome instability. The key to maintaining genome stability is in the function of these DNA repair proteins that regulate and maintain the pathways [1, 3, 5]. Understanding how and where proteins function in DNA repair pathways will allow better understanding of the mechanisms of human diseases and accelerated aging.

FANCONI ANEMIA

Interest in the FA pathway has been heightened because of several recent discoveries of new proteins tied to the pathway and the mechanisms of regulation of their activities. The pathway is of interest as well because several FA genes are also linked to breast cancer. FA is a recessive autosomal disorder characterized by developmental abnormalities, bone marrow failure, and a high predisposition to cancer. In general the disease has a worldwide

Abbreviations: BLM, Bloom's syndrome helicase; D-loop, displaced loop; FA, Fanconi anemia.

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prevalence of 1-5 per million and is found across all races and ethnic groups [7]. FA patients have an average life expectancy of 20 years and ultimately succumb to life threatening bone marrow failure, acute myeloid leukemia, or squamous cell carcinomas [7]. FA is also highly heterogeneous because it is a multigene disorder affecting the renal, cardiac, gastrointestinal, and reproductive systems [8]. To date, there are 13 complementation groups associated with the disease (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, and N) with 12 of them mapped on autosomal chromosomes and FANCB mapped on the X chromosome [9]. FANCA, C, and G are the most commonly found mutated in FA patients with a prevalence of 66, 10, and 9%, respectively [1, 3]. The cellular hallmark of FA is hypersensitivity to DNA crosslinking agents and high frequency of chromosomal aberrations pointing to a defect in DNA repair [8]. Increasing evidence suggests that the FA proteins work in a DNA-damage response pathway as signal transducers and processing machines [10].

FANCONI ANEMIA PATHWAY

The FA pathway closely interacts with other DNA repair pathways such as homologous recombination, nucleotide excision repair, or translesion DNA polymerases in response to genotoxic stress [8]. The FA proteins interact with multiple known DNA repair proteins including the breast cancer susceptibility proteins: BRCA1 and BRCA2, Bloom's helicase and RAD51 recombinase involved in homologous recombination, and MutLα involved in mismatch repair. Recently the three FA proteins FANCD1, FANCN, and FANCJ were found to be identical to the breast cancer susceptibility proteins BRCA2, PALB2, and BRIP1, respectively [9]. From these facts it is obvious the FA pathway is linked to several other DNA repair pathways that are important for maintaining genome integrity.

The FA proteins are divided into three groups: (1) FA core complex, (2) FA-ID complex, and (3) downstream members (table and figure). This classification is based on how the proteins work together to process blocked or damaged replication forks during S phase of the cell cycle [4]. During this phase, the DNA is duplicated at multiple replication forks. DNA lesions can stall the replication fork and cause activation of the S-phase checkpoint machinery. Recombination enzymes, in particular nucleases, are known to have high affinities for DNA replication intermediates and can directly process newly synthesized strands to create single-stranded DNA (ssDNA) regions [11-13]. These ssDNA regions recruit an ssDNA-binding protein known as replication protein A (RPA) whose binding can activate the cell cycle checkpoint [13]. The checkpoint ATR kinase subsequently binds to RPA–ssDNA complex and phosphorylates a number of factors including FA core complex members and the Chk1 kinase that further propagates the damage signal through phosphorylation and thus activation of other damage control proteins. Both ATRIP (the binding partner of ATR) and HCLK2 are recruited to regulate ATR stability. FAAP24 (part of the FA network but not shown to cause FA) forms a heterodimer with the FANCM helicase and associates with HCLK2 [4]. FANCM is part of the FA core complex and serves as a landing pad and a motor for the other members of the FA core complex composed of FANCA, B, C, G, E, F, L (table). Therefore, FANCM links the other FA core complex members to the stalled replication fork. The FA core complex was originally purified as a larger multi-complex called the BRAFT complex. This complex contains BLM, replication protein A, FA proteins, and topoisomerase IIIα [9]. BLM is a RecQ helicase implicated in the Bloom's syndrome cancer predisposition disorder [14, 15]. Topoisomerase IIIα works with BLM to resolve DNA intermediates [9].

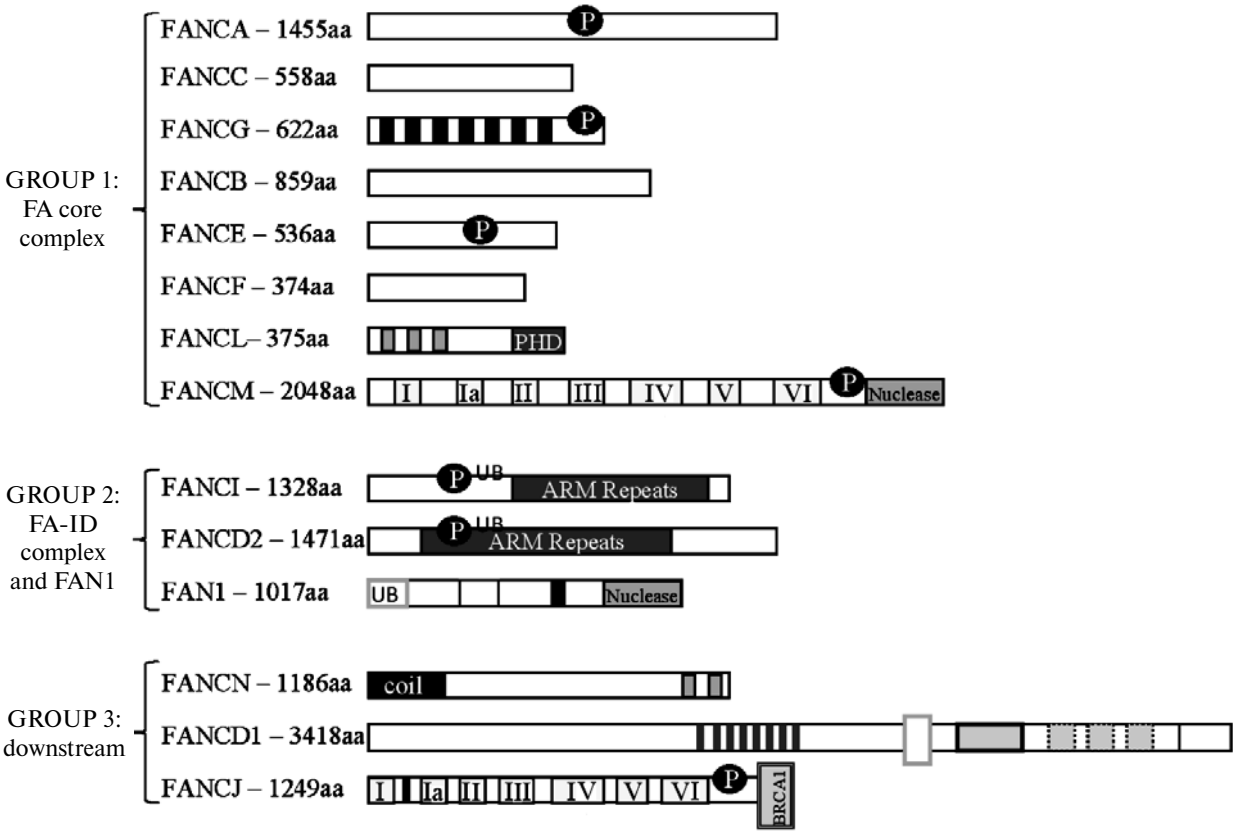
Once the FA core complex is recruited to the site of damage it acts as an E3 ligase to monoubiquitinate the FA-ID complex composed of FANCI and FANCD2. This monoubiquitination as well as phosphorylation by ATR causes the relocation of the FA-ID complex to nuclear foci containing the breast cancer susceptibility protein, BRCA1 [16]. It is thought that these foci are sites of DNA repair [9]. What happens after the FA-ID complex relocation to DNA repair foci is unknown, but there are currently two theories. The first theory is that FANCD2 recruits FANCD1/BRCA2 to facilitate homologous recombination-mediated repair. The second theory is that the complex promotes the release of the replicative DNA polymerases from the stalled fork and recruitment of the translesion polymerase REV1 to bypass DNA lesions. Recent evidence that will be discussed later on in this review suggests that the FA-ID complex along with the newly discovered FAN1 nuclease could directly carry out the repair of damaged DNA.

The first two groups of FA proteins (FA core complex and FA-ID complex) function together in the process of DNA damage repair. However, the third group of FA proteins is thought to work downstream and possibly independently of the FA core and FA-ID complexes (table and figure). These three proteins (FANCD1/BRCA2, FANCN, and FANCJ) are not required for the monoubiquitination of the FA-ID complex. A unique feature of these proteins that distinguish them from the other FA proteins is that heterozygous mutations at these loci lead to a high predisposition to breast cancer. These proteins have strong links to the homologous recombination pathway. For example, FANCD1/BRCA2 has been found in DNA damage foci with the RAD51 recombinase. FANCJ has been found in complexes with BRCA1 and the mismatch repair complex. Specific functions of this downstream group of FA proteins in DNA repair are

Domain structure and known activities of thirteen disease-causing FA proteins and the newly discovered FAN1 nuclease

FA protein	Domains/motifs/modifications	Activities
	Group 1: FA core complex	
FANCA	phosphorylated following DNA damage	important for incision activity of ERCC1-XPF to unhook a crosslink
FANCB	nuclear localization signal	
FANCC		implicated in telomere recombination, associates with BLM, important for ERCC1-XPF incision activity
FANCE	phosphorylated following DNA damage	links FANCL E3 ligase to FANCD2 (part of FA-ID complex)
FANCF		
FANCG	seven conserved TPR repeats, phosphorylated following DNA damage	scaffolding protein for ERCC1-XPF incision activity and for FANCD1/BRCA2, FANCD2, and XRCC3 for homologous recombination
FANCL	three WD40 repeats, PHD finger	putative E3 ligase for FA-ID complex
FANCM	seven conserved helicase motifs (I, Ia, II, III, IV, V, VI), endonuclease-like domain, phosphorylated following DNA damage	binds to HCLK2 and FAAP24 to recognize stalled replication forks, landing pad for FA core complex, unwinds and translocates DNA using helicase activity
	Group 2: FA-ID complex and FAN1	
FANCI	ARM repeats, conserved lysine is monoubiquitinated, phosphorylated following DNA damage, C-terminal DNA-binding domain	reciprocal activation with FANCD2 with recruitment to chromatin, binds with high affinity to Holliday junction and replication fork, linked to FAN1
FANCD2	ARM repeats, conserved lysine is monoubiquitinated, phosphorylated following DNA damage	reciprocal activation with FANCI with recruitment to chromatin, binds with high affinity to dsDNA ends and Holliday junctions, possible exonuclease helper of FAN1
FAN1	nuclease-like fold at C terminus, SAP DNA-binding domain, TPR motif, zinc-finger ubiquitin domain at N terminus	5′-3′ exonuclease activity, endonuclease activity, linked to FA-ID complex, linked to mismatch repair proteins
	Group 3: Downstream	
FANCN (PALB2)	two WD40 repeats at C terminus, coiled-coil motif at N terminus	regulates FANCD1/BRCA2 function, mediator of BRCA1 and FANCD1/BRCA2 interaction
FANCD1 (BRCA2)	eight BRC repeats in midsection, helical domain at C terminus, three OB-folds, TR2 motif	regulates RAD51 and DMC1
FANCJ (BRCH1/BRIP1)	seven conserved helicase motifs (I, Ia, II, III, IV, V, VI), iron-sulfur domain, BRCA1-interacting domain, phosphorylated following DNA damage	5′-3′ ATPase-dependent dsDNA unwinding activity for direct processing of damaged DNA, interacts with BRCA1 for DNA repair, interacts with TopBP1 and MutLα, linked to FANCD2 foci appearance

less clear, and more is known about their interacting partners. Similar to the FA core complex, ATR kinase phosphorylates BRCA1 and BRCA2 followed by their relocation to DNA damage sites where they further recruit other DNA repair proteins to mediate a homologous recombination-dependent restart of the stalled replication fork. FANCN is a mediator of BRCA2 and links it to BRCA1. FANCJ helicase has a BRCA1-binding domain



Domains and motifs of the thirteen FA proteins and the newly discovered FAN1. The proteins are grouped into FA core complex, FA-ID complex and FAN1, and a downstream complex based on their functions and interactions within the FA pathway

that allows the two proteins to form a complex that promotes homologous recombination. In fact, FANCJ might promote loading of BRCA1 or translesion bypass polymerases by disrupting unwanted protein–DNA complexes or DNA intermediates using its helicase activity. However, more data is necessary to clarify the function of the downstream FA proteins.

FA CORE COMPLEX

FANCL is E3 ubiquitin ligase. DNA damage that occurs during S phase of the cell cycle activates the FA core complex, which is made up of eight FA proteins: FANCA, B, C, E, F, G, L, and M, and two associated FA proteins (FAAP24 and FAAP100) [4, 17]. All members of the FA core complex are required in order to monoubiquitinate the FA-ID complex. Interestingly, of all the FA core members only FANCL has a recognizable E3 ligase domain that could attach the ubiquitin to protein targets. FANCL has three WD40 repeats. WD40 repeats are stretches of approximately forty amino acids terminating in a tryptophan-aspartic acid (W-D) dipeptide. These WD40 repeats are known to mediate multiprotein complex assembly and have been implicated in various processes including signal transduction and regulation of the cell cycle [18]. FANCL also contains a PHD finger domain (plant homeo domain) that is implicated in protein–protein interactions. The PHD domain, which encodes a zinc finger motif, is found in over 400 eukaryotic proteins and functions in chromatin remodeling processes [19]. Recent studies suggest that the PHD domain acts as an E3 ligase to regulate protein targets [20]. In fact, recombinant FANCL protein was shown to possess ubiquitin ligase activity *in vitro*, and mutations in this PHD domain failed to ligate ubiquitin [18]. If FANCL is the only FA core complex protein with E3 ligase activity, then why are the other FA core members necessary for the monoubiquitination of the FA-ID complex? One possible answer is that the other core members have a role in delivering the putative E3 ligase to the target FA-ID complex. Indeed, the core FA members show interdependence in their functions. For example, FANCE forms a stable complex with FANCC, but not with the disease-causative FANCC mutant. Failure of FANCE to form a complex with FANCC impairs FANCE accumulation in the nucleus for the subsequent repair of DNA [21].

FANCM helicase. FANCM protein is one of few FA proteins that directly interact with DNA [22]. FANCM is a helicase that translocates along the DNA and relocates the FA core complex to the site of damage. In HeLa cells, depletion of FANCM by siRNA led to loss of chromatin localization of FANCA, G, and E, further confirming the role of FANCM in directing the FA proteins to DNA [23]. FANCM is a member of the superfamily 2 helicases and as such contains in the N-terminal part the seven conserved helicase motifs including the Walker A and Walker B motifs that bind and hydrolyze Mg^{2+}-ATP. The helicase motifs define the two tandem RecA-like folds (RecA-like lobes 1 and 2), which constitute the "core" or the minimal translocation motor that is responsible for conversion of chemical energy of ATP into protein conformational changes and, consequently, for the mechanical motion [24]. The FANCM gene homologous to the archaebacteria protein Hef (helicase-associated endonuclease for fork-structured DNA) is responsible for maintaining replication fork stability [23]. Hef also contains a third "nuclease" domain that is flanked by a helix–hairpin–helix motif. Hef uses this domain to homo-dimerize and cleave branched DNA substrates. Human FANCM as well as the eukaryotic orthologs including *Schizosaccharomyces pombe* (Fml1) and *Saccharomyces cerevisiae* (Mph1) either lack this nuclease domain completely or have critical residues deleted so that they do not have nuclease activity [22, 25]. Both Fml1 and Mph1 have DNA-dependent ATPase activity that strongly depends on availability of a 3′single-stranded DNA overhang in the DNA structure, indicating a preference for 3′-5′ polarity in DNA unwinding [4]. Fml1 and Mph1 can unwind dsDNA, four way DNA junctions, or DNA replication forks [26]. In contrast, FANCM does not have canonical helicase activity and is unable to unwind any DNA substrates. *In vitro*, human FANCM is able to displace a third, shorter DNA strand bound to duplex DNA in the triple-helix displacement assay, most likely because of its ability to translocate on DNA and not because of canonical, unwinding helicase activity [26, 27]. FANCM is also able to drive branch migration of Holliday junctions (important intermediates in homologous recombination) and replication forks in an ATP hydrolysis-dependent manner, exchanging one DNA strand for the other by the stepwise breakage and reformation of base pairs [28, 29].

FANCC and telomere recombination. The rest of the FA core members function together to signal DNA damage and recruit other DNA repair proteins. For example FANCA, E, and G are phosphorylated following DNA damage [9]. FANCC and FANCG may not only be involved in FA core complex signaling but also in regulating homologous recombination. FANCC and FANCG knockout DT40 chicken cells were assayed for homologous recombination repair using a modified GFP (green fluorescent protein) gene that contains two differentially mutated GFP genes inserted between a selection marker. DNA cleavage at a unique restriction site in one GFP gene can be repaired via a homologous recombination event that results in restoration of the intact GFP gene encoding GFP protein that can be detected by flow cytometry [30]. In both FANCC and FANCG knockout cell lines, homologous recombination was reduced [31-33]. FANCC may also be specifically related to telomere recombination because $FANCC^{-/-}$ murine bone marrow cells showed telomere shortening compared to the wild-type cells, even though in both cell lines telomerase levels were normal. Furthermore, blood cells derived from FA patients had shorter telomeres than control blood cells. Besides telomere recombination, FANCC may act as a scaffolding protein for the BRAFT complex, which includes the BLM helicase. More specifically, FANCC and BLM may function together to regulate sister chromatid exchange, as it is increased in double knockout chicken DT40 cells. Further evidence for an association between FANCC and BLM was shown in mice with the deletion of FANCC leading to reduced levels of BLM [34]. It is difficult to characterize FANCC because like FANCF, no known recognizable domain or motif has been discovered.

FANCG scaffolding protein forms two complexes. As stated earlier, it is also thought that FANCG has roles outside FA core complex formation. FANCG contains seven tetratricopeptide repeats (TPR), which are stretches of 34 amino acids that form helix–turn–helix structures, and these are thought to mediate protein–protein interactions. It is thought that FANCG uses these motifs to form different complexes depending on the type of repair needed, either homologous recombination-mediated repair of a DNA double-strand break or the unhooking of a crosslink [35]. In order to perform the different repair reactions, FANCG forms two different complexes—one that works specifically for crosslink repair of replication forks and another complex that participates in homologous recombination-mediated repair. Remodeling of crosslinks at replication forks involves multiple steps including cleaving the DNA at the crosslink and creating a double-strand break, incision and unhooking of the crosslink, DNA lesion bypass by translesion DNA polymerases, excision of the crosslink, and finally recruitment of homologous recombination proteins to repair the double-strand break and restart the replication fork [36].

The unhooking of the crosslink is an important step that is performed by a structure-specific endonuclease known as ERCC1-XPF (excision repair cross-complementing rodent repair deficiency complementation group 1 and xeroderma pigmentosum complementation group F). The endonuclease is involved in the nucleotide excision repair pathway and is thought to work in crosslink repair by cleaving at the 5′ end of the junction [37]. Recent studies have shown that FANCA, C, and G are important for the incision activity of ERCC1-XPF.

Patient-derived FANCA, B, C, D2, F, and G cell lines were unable to produce incisions to cut out crosslinked DNA [38]. When wild-type FANCA, C, and G were added back to the respective deficient cell lines, the ERCC1-XPF produced incisions went back to normal levels. This interaction with ERCC1-XPF endonuclease is strongly dependent on 1, 3, 5, and 6 of the eight TPR motifs present in FANCG, providing further evidence that FANCG is a scaffolding protein. The TPR motifs are also required for FANCG to bind to FANCF and FANCA. FANCG probably functions to bring these FA proteins as well as ERCC1-XPF together for incision of a crosslink [36].

The second complex that FANCG forms involves FANCD1/BRCA2, FANCD2, and XRCC3 (ability to complement X-ray sensitivity). XRCC3 is one of the five RAD51 paralogs, which are known to have roles in homologous recombination [35, 39]. RAD51 is the central homologous recombination protein that promotes the search for homologous chromosomes and the subsequent strand-exchange reaction in order to repair a DNA double-strand break. Sequence analysis found five proteins with ATP-binding domains very similar to those of RAD51; the specific functions of these analogs are currently unknown. The five paralogs have DNA-dependent ATPase activity with a preference for single-stranded DNA. In particular, XRCC3 has been shown to physically interact with RAD51 and is required for RAD51 foci formation following DNA damage [39-41]. Interactions with XRCC3 ties FANCG to a role in homologous recombination. This and other data suggest that the FA pathway works by engaging other DNA repair pathways, including homologous recombination, for the repair of damaged DNA.

Formation of the complex depends on the phosphorylation of FANCG at serine seven and is independent of FA core complex formation. The phosphorylation of FANCG is not only required for the direct interaction of FANCG with the other members of the complex, but also for the interaction between the other members of the complex. In CHO (Chinese hamster ovary), human fibroblast, and lymphoblast cell lines where *FANCG* was mutated, the FANCD2-FANCD1/BRCA2 complex failed to co-precipitate. However, when wild-type FANCG was expressed in these cells the co-precipitation of FANCD2-FANCD1/BRCA2 was restored. In FANCA, C, E, or F mutated cells lines, the monoubiquitination of the FA-ID complex failed but the FANCD2-FANCD1/BRCA2 complex was still formed. This indicates that complex formation is dependent on FANCG alone and not on the FA core complex or its ability to monoubiquitinate the FA-ID complex. Once this complex forms, it participates in homologous recombination through XRCC3, which is one of the five RAD51 paralogs known to have roles in homologous recombination [35, 39]. Gene knockouts were performed in chicken DT40 cells and showed that XRCC3 deficient cells were more sensitive to crosslinking agents than the FANCG knockouts. The FANCG/XRCC3 double knockout displayed the same phenotype as the XRCC3 single knockout, thus indicating that FANCG and XRCC3 participate in the same pathway.

The proposed role of XRCC3 in homologous recombination is based on its sequence homology that it shares with RAD51 and on DNA damage sensitivity and recombination deficiency of *XRCC3*$^{-/-}$ cells. XRCC3 has a 20-30% sequence identity with RAD51 with the greatest conservation in its ATP-binding domain [42]. Mutations in the RAD51 paralogs, including XRCC3, lead to an increase in spontaneous chromosomal aberrations similar to that observed in FA cells [42]. RAD51C, another RAD51 paralog, is the direct binding partner of XRCC3. Biallelic mutations in RAD51C lead to FA-like disorders, whereas monoallelic mutations are associated with breast and ovarian cancer similar to mutations in some of the FA genes [43]. Also, similar to FANCM and FANCI, complexes formed by the RAD51 paralogs are known to have high affinities for such DNA structures as Holliday junctions and replication forks [44, 45]. Thus, FANCG could function first with the FA core complex to promote removal of the crosslink and later as a scaffolding protein to recruit the homologous recombination proteins to repair the double-strand break.

FA-ID COMPLEX

FANCI and FANCD2. Once the FA core complex is assembled at a stalled replication fork, it works along with the PI-3 kinase, ATR, to activate the FA-ID complex composed of FANCI and FANCD2. Both FANCI and FANCD2 are highly conserved among eukaryotes from humans to slime molds, but no orthologs have been found in yeast [46]. The two proteins have 13% identity and 20% similarity to one another and have a reciprocal activation relationship. For example, if FANCI is depleted there is decreased ubiquitination and foci localization of FANCD2 after DNA damage, as is true in the reciprocal relationship. Both proteins contain ARM repeats, which are $\alpha-\alpha$ superhelix folds that pack together tightly to form a hydrophobic core important for protein–protein interactions. There is also a conserved lysine that is monoubiquitinated in both proteins in order to activate them. This monoubiquitinated and phosphorylated complex is implicated in maintaining genome stability because gene knockdown experiments of FANCI and FANCD2 reduced homologous recombination in cells [46]. Also, FANCI depleted cells had increased levels of γ-H2AX phosphorylation, which is an indicator of double-strand break repair, and this was seen in unirradiated cells, indicating that FANCI maintains genomic stability by preventing spontaneous damage.

DNA binding studies showed that FANCI has the highest binding affinity for the Holliday junction, then replication fork, and finally duplex DNA, and that the C-terminal portion of FANCJ contained the DNA-binding domain [47]. Differently, FANCD2 showed a high binding affinity for double-stranded DNA ends and Holliday junctions. It is also possible that FANCD2 forms a multimer on DNA, because binding at the DNA ends requires greater than 65 bp to be available. FANCD2 binds DNA regardless of monoubiquitination (or phosphorylation), so the purpose of the monoubiquitination remains unclear [48].

FAN1 nuclease associated with FA-ID complex. Once the FA-ID complex is recruited to sites of DNA damage the complex promotes the repair of DNA lesions through nucleolytic processing and translesion DNA synthesis, but how it does this is still unknown [49]. It is thought that the complex recruits ubiquitin-binding proteins that are involved in DNA damage control. Recently, in a genetic screen probing for proteins involved in DNA interstrand crosslink repair, a nuclease that associated with FA proteins was identified [49, 50]. The nuclease, named FAN1 (Fanconi-associated nuclease 1), has 5′-3′ exonuclease activity and endonuclease activity and could be the key factor involved in FA-ID complex repair. FAN1 has several domains that link it to DNA repair activities. For example, it has a nuclease-like fold at its C-terminal and a SAP domain that is generally considered a DNA-binding domain of proteins involved in chromosomal organization. Similarly to FANCG, it has a TPR motif for protein–protein interactions in the midsection, and lastly it has a zinc-finger ubiquitin-binding domain at its N-terminus (UBZ). HA-FAN1 was purified from 293TREX cells (stably express the tetracycline repressor protein), and it was found to interact with the mismatch repair proteins MLH1, MLH3, PMS1, and PMS2, thus implicating FAN1 in mismatch repair. Because FANCJ, a member of the downstream FA pathway, also interacts with the mismatch repair proteins, it is possible that FAN1 nuclease works together with this complex as well, which would tie the downstream pathway together with the other two FA groups.

To determine the localization of FAN1 after DNA damage, cells expressing GFP-labeled FAN1 were studied. The cells were exposed to laser microirradiation, which leads to DNA damage tracks and FAN1 localized to these sites along with the DNA damage marker γ-H2AX. Because of the ubiquitin-binding domain of FAN1, it is thought that FAN1 could possibly interact with the monoubiquitinated FA-ID complex. Cells damaged by the crosslinking agent mitomycin C showed that FAN1 localized to damage foci along with FANCD2. Mutagenesis studies further showed that this localization was largely dependent on an intact ubiquitin-binding domain. Ubiquitin-deficient mutants of FANCI-FANCD2 moderately interacted with FAN1, whereas the monoubiquitinated complex interacted strongly with FAN1 [50]. Cells depleted of both FANCI and FANCD2 and treated with mitomycin showed that GFP-FAN1 was no longer able to form damage foci [49, 50]. In a cell line lacking FANCD2, FAN1 foci failed to appear, but after adding back monoubiquitinated-FANCD2 the FAN1 foci could be reconstituted. FAN1 foci formation also depends on monoubiquitination of FANCD2 because FANCD2 mutants unable to be ubiquitinated could not restore FAN1 foci in these cells.

Recent immunoprecipitation experiments indicate that FAN1 foci formation is dependent on FANCD2 monoubiquitination [50]. It was proposed that FAN1 uses its ubiquitin-binding domain to bind to the monoubiquitinated FANCD2 that is already bound to monoubiquitinated FANCI [51]. It was also proposed that FAN1 and FANCD2 process damaged DNA together, and FANCD2 acts as an exonuclease helper. Recent studies found that both chicken and human FANCD2 were able to degrade the DNA in the 3′-5′ direction in the presence of Mg^{2+}, even though sequence studies have not identified an exonuclease domain in FANCD2 [52]. More studies are needed to characterize the exonuclease activity of FANCD2 and its interaction with FAN1 during DNA repair.

Once FAN1 binds to monoubiquitinated FANCD2-FANCI, the complex is recruited to stalled replication forks where FAN1 uses its nuclease activities to process the damage. FAN1 was shown to have high exonuclease activities on 3′ flap substrates and nicked substrates as well as an endonuclease activity on replication forks, 5′ flaps, and nicked circles. FAN1 could use its nuclease activity to unhook the crosslink at a stalled replication fork. FAN1 could also function later on to process the ends of the double-strand break helping to initiate DNA repair via homologous recombination [53]. Using the repair of I-Sce-I induced DNA double-strand breaks, it was found that in cells depleted of FAN1 homologous recombination repair was reduced by 50-60% [54]. In repair of DNA double-strand breaks the ends at the break are resected to create 3′ overhangs, and RPA protein coats the newly exposed single-stranded DNA. Although it seems possible that FAN1 is the nuclease that creates the 3′ overhangs, current data indicate its role at later steps of homologous recombination. Thus, after resection of the DNA double-strand break, homologous recombination protein RAD51 forms a nucleofilament on the ssDNA to search for homologous DNA to repair the break. Following a double-strand break, RAD51 foci formation was increased in both wild-type and FAN1-depleted cells, indicating that DNA resection was taking place. However, FAN1-depleted cells had sustained RAD51 foci formation compared to wild-type cells, suggesting that FAN1 participates in late steps of homologous recombination and in some way works to clear RAD51 after repair is complete [54]. These results tie the FAN1 function to homologous recombination as well as to the FA pathway.

DOWNSTREAM MEMBERS

The remaining members of the FA proteins—FANCD1/BRCA2, FANCN, and FANCJ—are thought to work in a downstream or possibly separate pathway compared to the proteins that form the FA core and FA-ID complexes. This is because cells lacking any of these downstream proteins still show monoubiquitinated FANCI and FANCD2. In contrast, cells lacking any of the FA core members show no monoubiquitination of the FA-ID complex. Also, members of this group have been linked to a predisposition to breast cancer; interestingly, breast cancer is associated with heterozygous mutations in these genes, whereas homozygous mutations cause FA [9].

FANCN regulates FANCD1/BRCA2. FANCN was originally called PALB2 (partner and localizer of BRCA2) because it interacts and regulates the breast cancer tumor suppressor protein BRCA2, which is now known to be identical to FANCD1. FANCN mutation identified in one FA patient allowed for normal monoubiquitination of the FA-ID complex. HeLa cells, when FANCN expression was reduced by siRNA, displayed hypersensitivity to DNA damaging agents, a hallmark of FA, and mislocalized RAD51 and BRCA2 proteins [55]. When FANCN expression was returned to normal, FANCD1/BRCA2 and RAD51 foci formation was restored and the cells were no longer hypersensitive to DNA damaging agents, e.g. mitomycin C.

FANCN has two C-terminal WD40 repeats and a conserved N-terminal coiled-coil motif [56]. The WD40 repeats promote FANCN binding to the N terminus of FANCD1/BRCA2. FANCN controls the chromatin localization, the checkpoint function, and the homologous recombination repair function of FANCD1/BRCA2 [57]. Mutations in BRCA2 that lead to breast cancer disrupt binding of the protein to FANCN and also cause homologous recombination repair defects, thereby providing evidence for functional importance of the BRCA2-FANCN binding [57]. Recent studies have further implicated FANCN as a mediator protein of not only FANCD1/BRCA2 but also BRCA1 in DNA repair [56]. In FANCN deficient cells, the interaction between BRCA1 and BRCA2 was decreased, but upon adding back FANCN the association was restored. Similar to FANCN, BRCA1 also contains a conserved coiled-coil domain, and mutations in this domain on either protein prevent the FANCN-BRCA1 interaction. BRCA1 controls the relocation of FANCN and BRCA2 in response to DNA damage because cells that contained a BRCA1 truncated mutant were unable to form damage foci containing BRCA2 or FANCN after ionizing radiation treatment [56].

Mouse knockout studies conducted with FANCN showed that a double knockout of the gene resulted in embryonic lethality similar to both BRCA1 and BRCA2 double knockout mice. Before embryonic death, there was a defect in cell proliferation, likely due to inability to repair accumulating DNA lesions [58]. Based on the mouse and biochemical studies, FANCN is recruited to damage sites where it forms oligomers that recruit BRCA2 and regulate its function in replication checkpoint control, homologous recombination repair, and its interaction with BRCA1. Therefore FANCN, similar to upstream FANCG, acts as a scaffolding protein to recruit BRCA2 and BRCA1 [58].

FANCD1/BRCA2 recombinase regulator. BRCA1 and FANCD1/BRCA2 are tumor suppressor proteins that are mutated in breast cancer, which is one of the most common cancers in the US. BRCA1 and BRCA2 mutations are associated with 5% of breast cancers [59]. BRCA2 mutations are associated with a 50% risk in developing the cancer before age 50 [60]. Differently than BRCA1, BRCA2 not only increases risk for breast cancer in women, but also male breast, pancreas, and prostate cancers [59]. It was found that the breast cancer proteins are also involved in the FA pathway. Thus, disruption of BRCA1 leads to a loss in DNA damage foci containing FANCD2. It was then found that fibroblast cells from the FANCD1 complementation group have biallelic mutations in BRCA2 and that wild-type BRCA2 restored the resistance of the cells to mitomycin C [61]. This led to the discovery that FANCD1 and BRCA2 are one and the same protein.

FANCD1/BRCA2 is large protein of 3418 amino acids and has recognizable domains indicating it could function as a large scaffolding protein [59-62]. FANCD1/BRCA2 contains eight BRC repeats in the midsection of the protein, which are degenerate repeating units of about 70 amino acids that contain a core of 25 amino acids [60]. Multiple studies indicate that BRC 1-4 are responsible for strong interactions with RAD51 recombinase, and this is why FANCD1/BRCA2 is thought to be a recombinase regulator in the cell [60]. Studies have indicated that the BRC repeats work to stimulate RAD51 loading and strand exchange on single-stranded DNA but prevent nucleation on double-stranded DNA [57, 63, 64]. The C-terminal portion of FANCD1/BRCA2 contains a helical domain, three oligonucleotide/oligosaccharide-binding folds (OB-folds), and a TR2 motif that is highly conserved in vertebrate FANCD1/BRCA2 proteins [60, 62]. The OB-folds form a region for ssDNA binding and strongly resemble motifs seen in both prokaryote and eukaryote ssDNA-binding proteins like RPA and SSB.

An acidic protein, DSS1, is known to bind to the helical portion, as well as OB-folds 1 and 2, in order to stabilize FANCD1/BRCA2. When DSS1 protein was knocked down in cells, FANCD1/BRCA2 protein levels decreased due to degradation, and the cells then became hypersensitive to DNA damaging agents [65]. FANCD1/BRCA2 also regulates RAD51 through its TR2

motif and cannot bind to RAD51 if a key serine 3291 residue is phosphorylated [66]. The TR2 motif is found in the C-terminal portion (amino acids 3265-3330) and contains five conserved Ser/ThrPro repeats and an Arg-x-Leu binding motif. The Arg-x-Leu motif is a binding region for cyclin-dependent kinases, and the Ser/ThrPro repeats are potential targets for cyclin-dependent phosphorylation in order to control FANCD1/BRCA2 function during the cell cycle [66]. During S phase of the cell cycle, there are low levels of phosphorylation of the TR2 region in FANCD1/BRCA2 allowing for the protein to interact with RAD51 in the case of replication-induced DNA damage. As the cell cycle progresses to the G2/M phase, phosphorylation of the TR2 region increases. However, if DNA damage occurs FANCD1/BRCA2 is no longer phosphorylated and can interact with RAD51 to participate in homologous recombination-mediated DNA repair. This also explains why C-terminal truncating mutations of FANCD1/BRCA2 lead to cancer; the truncated FANCD1/BRCA2 is unable to interact and load RAD51 onto damaged DNA for its subsequent repair [66].

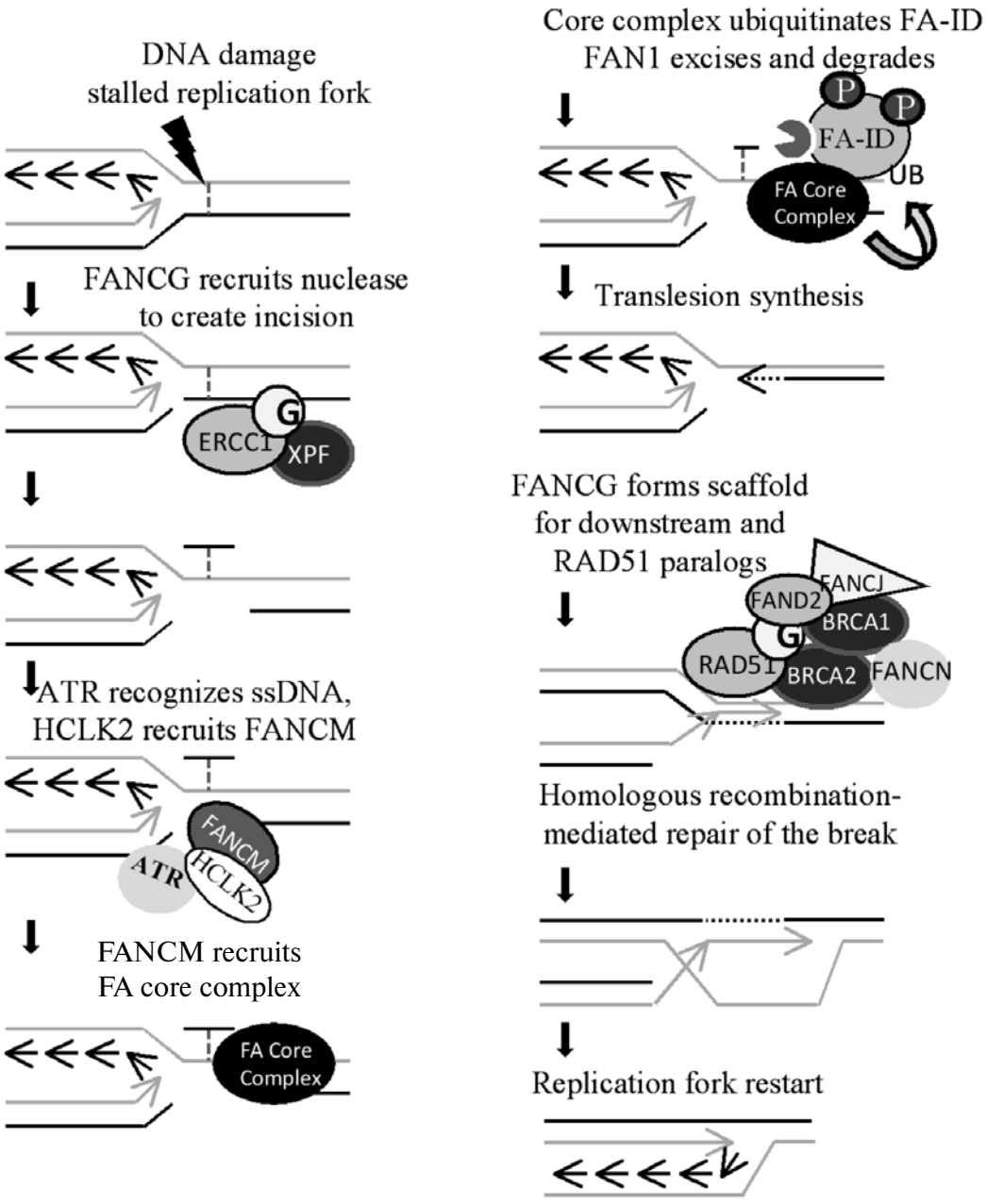
FANCD1/BRCA2 appears to be a universal regulator of recombinases because it is also involved in regulating the meiosis-specific recombinase DMC1. BRH2, the *U. maydis* homolog of FANCD1/BRCA2, is required for homologous recombination during mitosis as well as for meiosis recombination that results in the formation of viable haploid progeny [60]. Mutations in the *C. elegans* FANCD1/BRCA2 homolog resulted in meiosis defects, and one of the clinical manifestations of FA is reduced fertility. It was found that DMC1 protein interacts with human FANCD1/BRCA2 at two sites. The first weaker site was mapped to the same C-terminal TR2 motif that is involved in RAD51 binding; RAD51 was able to compete with DMC1 for binding to this site. The second stronger site that contains conserved KVFVPPFK motif, named PhePP motif, showed DMC1-specific binding. This site located between the last BRC repeat and the helical domain on the C terminus is highly conserved among the mammalian homologs [67]. These data indicates that FANCD1/BRCA2 plays a role in maintaining genome stability through regulation of a recombinase; mutations in FANCD1/BRCA2 prohibit interactions with DNA repair proteins causing FA or breast cancer.

FANCJ helicase. The third member of the downstream pathway is FANCJ, a DNA helicase that is hypothesized to operate at the interface between the FA and homologous recombination pathways [8-10, 68-73]. The specific role of FANCJ in maintaining genomic integrity is currently unknown. Because of its helicase activity, it likely processes DNA repair intermediates directly. FANCJ is evolutionarily conserved in all eukaryotes; its orthologs are found in all eukaryotes, from yeasts to humans. It is one of only a few FA proteins that interact directly with damaged DNA [73]. Importantly, loss of FANCJ compromises the repair of DNA double-strand breaks [73]. FANCJ is important for DNA crosslink repair, homologous recombination, mismatch repair, and replication checkpoint control [74, 75]. FANCJ likely has an additional function, independent of the FA core pathway, because double mutations of FANCC (part of the FA core complex) and FANCJ have an additive effect on cell sensitivity to crosslinking agents [73]. More importantly, mutations in FANCJ, similar to other downstream members, were found in a subset of patients with early onset breast cancer, indicating FANCJ is also a tumor suppressor. The helicase activity of FANCJ is critical for its tumor suppressor function. All breast cancer and FA-causing mutations identified so far, P47A, M299I, and A349P, severely diminish the helicase activity of FANCJ [10].

FANCJ has a 5′-3′ ATPase-dependent dsDNA unwinding activity. The helicase activity requires DNA substrates with an ssDNA region of a minimal length of 15 nucleotides and an optimal length of 35 nucleotides [76]. *In vitro* studies show that FANCJ has binding and unwinding preferences for forked substrates. FANCJ is able to disrupt D-loop structures that represent the three-strand invading intermediate formed by RAD51 during homologous recombination [72]. Furthermore, FANCJ is able to displace RAD51 from single- and double-stranded DNA indicating a possible regulatory role in homologous recombination [72, 77]. FANCJ belongs to the DEAH family of superfamily 2 helicases and as such contains the seven conserved motifs. It has the Walker A motif that binds ATP and the DEAH box in the Walker B motif that binds Mg^{2+}. The seven motifs are a signature if the conserved protein fold that hydrolyzes ATP and promotes translocation of the helicase along DNA and DNA unwinding. FANCJ has a conserved iron–sulfur cluster located between the two ATP-binding motifs that is thought to preserve the activities of the two flanking domains and help the helicase to distinguish between single- and double-stranded DNA [69, 78]. An FA mutation at this cluster still allows ATP hydrolysis and DNA translocation but prevents unwinding of the DNA. It was also shown that similar iron–sulfur mutations in the related helicase XPD cause the disease xeroderma pigmentosum, demonstrating the importance of an intact iron–sulfur cluster for functional helicase activity [78]. The XPD helicase was shown to bind iron ion using an iron chelation assay, and it is likely that FANCJ binds iron as well. The crystal structure of XPD shows that the iron–sulfur domain and an Arch domain in the first helicase motif form a channel for single-stranded DNA. In this case the iron–sulfur domain is proposed to have a role in breaking a DNA duplex, which is consistent with the observation that ATPase and DNA translocase activity are still intact in iron–sulfur mutants, but unwinding activity is abolished. It was also proposed that the iron–sulfur domain may act as a sensor for DNA damage

or that the activity of the helicase can be controlled by its redox state [79, 80]. The C terminus of FANCJ contains the BRCA1-interacting domain that binds directly to the BRC repeats in BRCA1 [10].

FANCJ and the tumor suppressor BRCA1 interact physically and colocalize at sites of DNA damage. BRCA1 and FANCJ have also been found in a complex with other repair proteins including TopBP1 and MutLα. Knockdowns or helicase-inactivating mutants of FANCJ lead to the accumulation of unrepaired DNA breaks in human cells. Homologous recombination studies conducted separately with defective BRCA1 or FANCJ have



The FA signaling pathway of DNA damage repair. DNA interstrand crosslinks cause stalling of DNA replication forks. FANCG recruits endonucleases to create incisions around the crosslink. This creates a double-strand break and formation of ssDNA. The ssDNA activates ATR in a complex with its binding partners ATRIP and HCLK. FANCM helicase is directly recruited by HCLK and serves to stabilize the fork and recruit the FA core complex to the site of damage through its DNA translocase activity. The FA core complex activates the FA-ID complex through monoubiquitination of both FANCI and FANCD2. ATR phosphorylates the FA-ID complex as part of the activation step. FA-ID complex in conjunction with FAN1 nuclease excises the crosslink and makes room for translesion polymerases to bypass the DNA lesion. FANCD2 of the FA-ID complex interacts with FANCG and members of the downstream FA pathway to initiate homologous recombination-mediated repair of the double-strand break and restart of the DNA replication fork

shown that there is a 10-fold reduction in homologous recombination in both cases. FANCJ is phosphorylated during S phase of the cell cycle and subsequently localizes to chromatin. Cells lacking FANCJ and exposed to DNA damage are not able to complete replication [73]. Previous studies suggest that the downstream proteins function separately from the upstream FA core complex and FA-ID complex, but recent studies have found that FANCJ could play a role in forming FANCD2 foci following DNA damage [81]. The study found that FANCJ-deficient cells showed fewer FANCD2 foci formation following DNA damage. It also found that reintroduction of FANCJ led to brighter FANCD2 foci appearance. The recruitment was not reciprocal in that FANCD2 depletion did not prevent FANCJ foci from forming. In the same study, BRCA1 depletion led to a reduction of FANCD2 foci formation to the same extent as FANCJ depletion. So far an actual physical link between FANCJ and FANCD2 has not been found, which may suggest that FANCJ indirectly recruits FANCD2 to damage foci. As a helicase FANCJ may process damaged DNA directly and create a binding site for FANCD2 to the damaged chromatin. Furthermore, BRCA1- and FANCJ-deficient cells show a similar decrease in FANCD2 foci formation indicating that they function in the same pathway. It is possible that FANCJ may link BRCA1 to FANCD2 [81]. Thus, FANCJ is involved in multiple pathways by possibly linking them together and by directly processing DNA using its helicase activity.

FA is a highly complicated and heterogeneous disease caused by mutations in thirteen genes that comprise the FA pathway. Continuing research has shown that the FA pathway is interrelated with other pathways in order to repair DNA damage. However, the main function of the FA pathway is in DNA damage signaling and remodeling of stalled replication forks (Scheme) [82]. The hallmark phenotype of FA cells is hypersensitivity to DNA crosslinking agents because interstrand crosslinks inhibit DNA replication and transcription. The upstream FA core complex functions mainly as a signaling complex that accumulates at a damaged replication fork and recruits the FA-ID complex. FANCM serves to anchor the core complex and translocate along the DNA to the specific site. Other members of the core complex serve as scaffolding proteins and signaling proteins of homologous recombination to promote the repair of the stalled fork. This model is consistent with a known requirement in multiple signaling pathways during repair of a stalled replication fork. Once the FA-ID complex is recruited to chromatin it recruits the FAN1 nuclease to help process and cleave out the actual DNA damage, again leading to formation of a double-strand break. This would lead to recruitment of homologous recombination proteins; FANCI and FANCD2 knockouts shown deficiencies in homologous recombination-mediated repair. Members of the downstream pathway are tied not only to FA but are responsible for a high predisposition to breast cancer. Previous theories hold the downstream members independent of the upstream FA members, but recent evidence suggests that this might not be the case. FANCJ appears to have some partial role in recruitment of the FANCD2 part of the FA-ID complex to DNA damage. The downstream members also link the FA pathway with breast cancer tumor suppressor proteins and play a role in homologous recombination. The downstream members may form a complex with FANCG and FANCD2 as well as with the RAD51 paralogs. This complex would perform double-strand break repair through homologous recombination and subsequent restart of the stalled replication fork.

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