

# Fanconi Anemia Proteins, DNA Interstrand Crosslink Repair Pathways, and Cancer Therapy

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**Abstract:** DNA interstrand crosslinkers, a chemically diverse group of compounds which also induce alkylation of bases and DNA intrastrand crosslinks, are extensively utilized for cancer therapy. Understanding the cellular response to DNA damage induced by these agents is critical for more effective utilization of these compounds and for the identification of novel therapeutic targets. Importantly, the repair of DNA interstrand crosslinks (ICLs) involves many distinct DNA repair pathways, including nucleotide excision repair, translesion synthesis (TLS), and homologous recombination (HR). Additionally, proteins implicated in the pathophysiology of the multigenic disease Fanconi anemia (FA) have a role in the repair of ICLs that is not well understood. Cells from FA patients are hypersensitive to agents that induce ICLs, therefore FA proteins are potentially novel therapeutic targets. Here we will review current research directed at identifying FA genes and understanding the function of FA proteins in DNA damage responses. We will also examine interactions of FA proteins with other repair proteins and pathways, including signaling networks, which are potentially involved in ICL repair. Potential approaches to the modulation of FA protein function to enhance therapeutic outcome will be discussed. Also, mutation of many genes that encode proteins involved in ICL repair, including FA genes, increases susceptibility to cancer. A better understanding of these pathways is therefore critical for the design of individualized therapies tailored to the genetic profile of a particular malignancy. For this purpose, we will also review evidence for the association of mutation of FA genes with cancer in non-FA patients.

**Keywords:** Fanconi anemia, DNA interstrand crosslinks, homologous recombination, DNA repair, DNA damage responses, chemotherapy, bifunctional alkylating agents, platinum compounds.

## INTRODUCTION

The majority of drugs currently used to treat cancer are DNA damaging agents [1-4]. Cells have multiple pathways, including mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), non-homologous end joining (NHEJ), translesion synthesis (TLS), and homologous recombination (HR), to repair DNA damage induced by these agents (reviewed in [3-6]). These repair pathways are regulated and coordinated by checkpoint networks governed by the ATM and ATR checkpoint kinases (reviewed in [5, 7-9]). Checkpoints, including the p53 tumor suppressor, also regulate cell cycle arrest and apoptosis in response to DNA damage [5, 10]. Importantly, cellular responses to DNA damage, involving DNA repair and checkpoint pathways, are critical determinants of therapeutic outcome.

A number of DNA damaging agents broadly utilized in cancer treatment induce DNA interstrand crosslinks (ICLs) [11-14]. These include bifunctional alkylating agents, such as nitrogen mustard derivatives, nitrosoureas, and alkyl sulfonates [11-14]. Among the nitrogen mustards which are utilized therapeutically are cyclophosphamide, chlorambucil, and melphalan [13-15]. Nitrosourea compounds include CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea; lomustine] and BCNU [1,3-bis(2-chloroethyl)-1-nitroso-urea; carmustine], and an important alkyl sulfonate is busulfan.

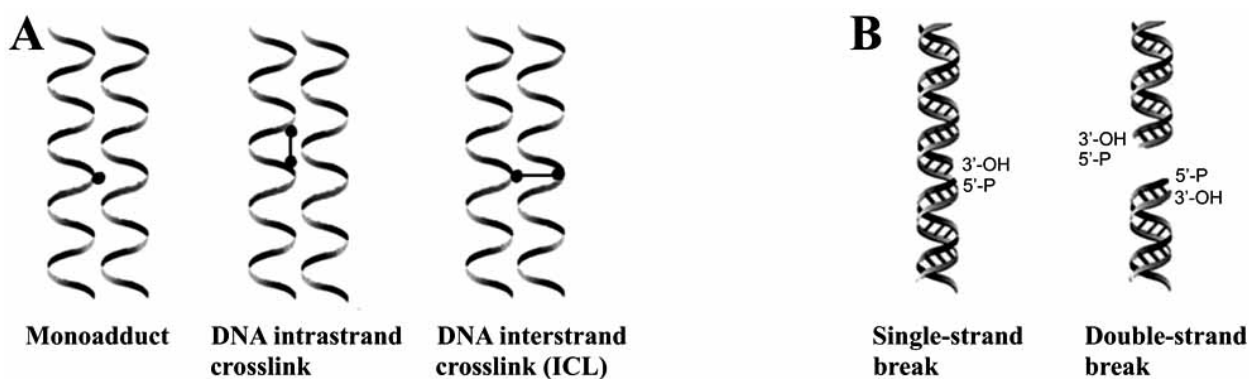
Other DNA interstrand crosslinkers include the natural product mitomycin C and platinum compounds, such as cisplatin, oxaliplatin, and carboplatin [16, 17].

The repair of ICLs involves multiple DNA repair pathways, including NER, TLS, and HR (reviewed in [12, 18, 19]). Since DNA interstrand crosslinkers are utilized so widely, the DNA repair pathways which respond to these agents are potentially important therapeutic targets. Also, DNA interstrand crosslinkers are mutagenic and can thereby yield secondary malignancies [13, 15, 20]. Because specific repair pathways can alter mutagenesis by these agents, and because cytotoxicity and mutagenicity may be modified differently by these DNA repair pathways, it is important to understand how ICLs are repaired.

Bifunctional alkylating agents can induce monoadducts and DNA intrastrand crosslinks, in addition to ICLs [12, 15] (Fig. 1A). In fact, in many instances, monoadducts predominate over ICLs [16]. Thus, the major therapeutic benefit for a specific agent could derive from monoadducts, DNA intrastrand crosslinks, or DNA interstrand crosslinks. In this review we will focus on the repair of ICLs, since this type of lesion, and not monoadducts or DNA intrastrand crosslinks, is believed responsible for the majority of cytotoxicity induced by bifunctional alkylating agents due to blockage of DNA replication [12, 14, 19].

Other forms of DNA damage, such as single and double strand DNA breaks, can be generated by other types of DNA damaging agents or as intermediates in the repair of damage induced by ICLs (Fig. 1B). The spectrum of DNA damage induced by a particular DNA interstrand crosslinker is an important consideration in therapeutic regimens involving

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**Fig. (1). Diagram of different types of DNA damage.** (A) Modification of a single strand of DNA by chemicals, such as alkylating agents, induces a monoadduct. Such adducts can lead to crosslinks, either within a single DNA strand (DNA intrastrand crosslink) or between two paired DNA strands (DNA interstrand crosslink). (B) Other types of DNA damage, some of which can be induced in the process of repairing DNA interstrand crosslinks, include single-strand (SSBs) and double-strand DNA breaks (DSBs).

combination therapy. Maximum benefit may derive from using agents which induce complementary spectra of DNA damage.

The study of human chromosome instability syndromes, and of certain cancer associated genes, has been critical for the identification of mechanisms involved in cellular responses to DNA damage [6, 7, 21]. Further, cell lines from patients with these diseases have characteristic patterns of sensitivity or resistance to specific DNA damaging agents. In cases where the responsible genes have been identified, characteristic sensitivities identify potential therapeutic targets. Importantly, Fanconi anemia (FA) is associated with hypersensitivity to agents that induce DNA interstrand crosslinks (ICLs) [19, 22-25].

In this review we will examine the repair of ICLs, with a focus on the role of Fanconi anemia (FA) proteins. We will begin with an overview of distinct pathways involved in the repair of ICLs. We will then consider FA genes and proteins in detail. Clinical and cellular phenotypes that are associated with FA will be reviewed to introduce FA genes and define the DNA damage response pathways in which FA proteins may be involved. What is currently known about the functions and interactions of the many FA proteins will then be examined in depth. The way this knowledge has identified screens and novel approaches for the therapeutic modulation of FA proteins will also be considered. Finally, current knowledge of somatically acquired mutation of FA genes in cancer will be described as a basis for personalized therapy [6]. The presence of FA gene mutations might define cases in which DNA interstrand crosslinkers may be particularly effective. Alternatively, tumors in which genes associated with ICL repair are intact might be treated by combining agents that induce ICLs with inhibitors of pathways that repair ICLs.

## REPAIR OF ICLs INVOLVES MULTIPLE DNA REPAIR PATHWAYS

Recent work has identified many of the steps involved in the repair of DNA interstrand crosslinks (ICLs) (reviewed in [12, 18, 19]). The proteins involved in ICL repair, in general, are required for resistance to ICLs and represent potential

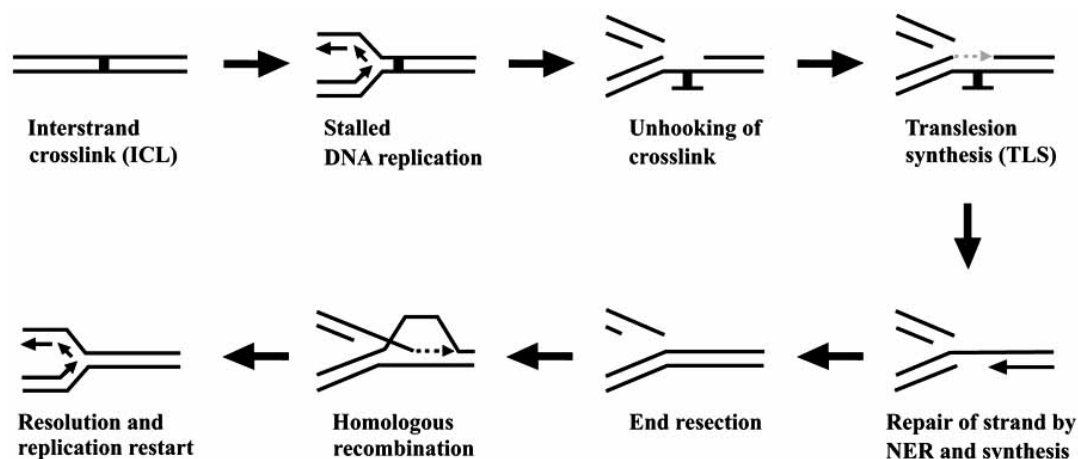
therapeutic targets. A highly simplified outline of these steps is presented in Fig. (2). The contribution of Fanconi anemia (FA) proteins to ICL repair, which is not well understood, will be considered in subsequent sections. It has been proposed that ICL repair is initiated when the replication fork encounters a lesion and becomes stalled [18, 19, 26]. This leads to signaling centered around the ATR checkpoint kinase, which is activated by replication stress [8, 27], and recruitment of the machinery for DNA repair. Since ATR and Chk1, which is activated by ATR-dependent phosphorylation, are required for homologous recombination (HR) [28, 29], these checkpoint kinases may also have an important role in regulating proteins involved at this late step of ICL repair.

Experiments with mammalian cell extracts have suggested that the MutS $\beta$  complex formed by the DNA mismatch repair (MMR) proteins, MSH2 and MSH3 [30], is involved in recognition of the ICL [31]. This is supported by a recent report, comparing a MSH2-deficient cell line and its genetically corrected counterpart, that demonstrated a role for MSH2 in resistance to, and in the repair of, psoralen-induced crosslinks in intact mammalian cells [32]. It should be noted, however, that the sensitivity of MMR-deficient cells to agents which induce ICLs is controversial, perhaps due to clonal differences in tumor-derived cell lines utilized in various studies [33].

The crosslink is unhooked by incision, both 3' and 5' of the lesion, by a process that involves the nucleotide excision repair (NER) proteins ERCC1 and XPF (Fig. 2) [12, 31, 34-36]. In effect, this generates a DNA double-strand break in the sister chromatid (Fig. 2). In accord with a role in ICL repair, cells which are deficient for either ERCC1 or XPF are sensitive to mitomycin C [37, 38].

The gap in the incised DNA strand is then filled in, and strand integrity restored, by the process of translesion synthesis (TLS). TLS bypasses the lesion and utilizes error-prone DNA polymerases (Fig. 2) [19, 26, 39]. The unhooked adduct on the other strand can then be removed by NER and strand integrity is restored by replicative DNA polymerases (Fig. 2) [19, 40].

The MRE11-RAD50-NBS1 (MRN) complex contains an exonuclease activity that can resect strands at the DNA dou-



**Fig. (2).** Schematic outline of the repair of DNA interstrand crosslinks (ICLs). A DNA interstrand crosslink blocks the progression of an advancing replication fork during S phase. This leads to signaling by the ATR checkpoint kinase, which recruits the machinery for DNA repair. Then the crosslink is unhooked, a process which involves incisions by endonucleases on both sides of the lesion on one DNA strand. This process requires the NER proteins ERCC1-XPF. This process also generates a DNA double-strand break which can initiate DNA repair by HR. To restore the integrity of the template strand for HR, the gap generated by unhooking the crosslink is filled in by the process of translesion synthesis (TLS) using bypass polymerases (indicated by a dotted gray line). The unhooked adduct is then removed by NER, or by spontaneous hydrolysis, and the gap is filled in by replicative DNA polymerases. End resection at the DNA double-strand break generates a 3' single-strand overhang which can pair with the sister chromatid that has been restored by TLS. End resection may involve exonuclease activity of the MRE11-RAD50-NBS1 complex. Strand invasion involves RAD51 and BRCA2. Following branch migration, resolution of Holliday junction recombination intermediates permits the restart of DNA replication, a process which may involve the Mus81-Eme1 endonuclease.

ble-strand break (Fig. 2) [6, 41]. End resection generates a 3' overhang that can pair with the sister chromatid to initiate HR. Accordingly, cells deficient for either MRE11 or NBS1 are sensitive to DNA interstrand crosslinkers [42-44]. Interestingly, deficiency for WRN, which is mutated in the chromosome instability disease Werner Syndrome, is also associated with hypersensitivity to ICLs [45, 46]. It has been reported that WRN interacts with the MRN complex [47], and that WRN has both 3'-5' exonuclease and helicase activities *in vitro* [48]. As such, WRN may be involved in end resection prior to strand invasion during HR.

Strand invasion is mediated by RAD51 oligomerization, in a process involving BRCA2 [49]. As would be expected, cells deficient for either RAD51 or BRCA2 are hypersensitive to DNA interstrand crosslinkers [50-53]. A role for BRCA2 in replication-coupled repair of ICLs, after unhooking of the lesion, has recently been confirmed *in vitro* using human cell extracts [40]. Homologous recombination restarts the stalled replication fork, in addition to repairing the DNA double-strand break generated by unhooking the ICL (Fig. 2) [19, 26, 54].

Reflecting the important role of HR in ICL repair, deficiency for other proteins involved in HR, including BRCA1, RAD54, and RAD51 paralogues, also results in hypersensitivity to ICLs [55, 56]. RAD54 is a member of the SWI2/SNF2 ATPase family which interacts directly with RAD51 (reviewed in [57]). The ATPase activity of RAD54 provides a potential target for small molecule inhibitors. Deficiency for any of the five RAD51 paralogues, RAD51- B, C, or D, and XRCC- 2 or 3, also results in hypersensitivity to ICLs, both in chicken DT40 cells and in mammalian cells [58-60]. The role of the RAD51 paralogues in HR is less well understood than that of RAD51 itself [61], but includes

a possible role for the RAD51C-XRCC3 subcomplex in the resolution of Holliday junctions [62].

The Mus81-Eme1 protein complex is an endonuclease that cleaves branched structures and which is required for HR. Mammalian cells deficient for Mus81 or Eme1 are hypersensitive to agents which induce ICLs [63, 64]. It has been reported that Mus81 and RAD54 physically interact [65]. The Mus81-Eme1 complex may play roles in conversion of the ICL to a DNA double-strand break [65] and in resolution of the Holliday junction at the completion of HR (Fig. 2) [66].

## FANCONI ANEMIA (FA) – CLINICAL AND CELLULAR PHENOTYPES

Fanconi anemia (FA) is a multigenic, clinically heterogeneous disorder associated with congenital abnormalities, progressive bone marrow failure, and a predisposition to cancer [22-24, 67, 68]. Hematopoietic defects are nearly universal in FA patients [68, 69]. Congenital abnormalities are heterogeneous and most commonly include skeletal defects, abnormal pigmentation, growth retardation, and microphthalmia [68, 70]. FA patients have an increased incidence of leukemia, primarily acute myeloid leukemia (AML), and various solid tumors. Squamous cell carcinomas of the head and neck are particularly prevalent, and occur at a young age, along with tumors of the esophagus, urogenital tract, skin (non-melanoma), breast, and lung [67].

Thirteen independent FA complementation groups have been defined and each of the corresponding genes (FANCA, B, C, D1, D2, E, F, G, I, J, L, M and N) have been identified [52, 71-87]. FA genes act in an autosomal recessive

manner, with the exception of FANCB, which is X-linked [74]. The shared clinical phenotypes of FA patients from different complementation groups, including hematopoietic abnormalities, congenital abnormalities, and predisposition to cancer, suggest that the encoded proteins cooperate in a shared pathway or process.

FA cells from different complementation groups display an abnormal response to DNA damage [22, 23], suggesting that this shared pathway is related to DNA damage responses. Cells from FA patients are characteristically hypersensitive to DNA interstrand crosslinking agents, such as mitomycin C (MMC) or diepoxybutane (DEB), as measured by cell survival assays [19, 22-25]. Other studies have suggested that FA cells have a broad sensitivity to agents which can induce DNA interstrand crosslinks, including cisplatin-related compounds and psoralen-UVA [88, 89]. While FA-D1 cells display a slight sensitivity to ultraviolet (UV) irradiation, FA cells, including FA-D1 cells, are not particularly sensitive to UV or ionizing radiation [88, 90, 91]. In general, FA cells have only a slight sensitivity to monoalkylating agents [90, 92].

Another cellular DNA damage phenotype which is shared between cells of different FA complementation groups is the induction of chromosome aberrations, including radial chromosomes and chromosome breakage, following treatment with MMC or DEB [22-24, 68]. This test is used clinically in the diagnosis of FA patients [93]. An additional phenotype that is shared by FA cells from different complementation groups is a characteristic accumulation in G2-M following treatment with agents which induce ICLs [85, 88, 94-98]. Together, the shared cellular phenotypes of cells from different FA complementation groups suggest that FA proteins have a specific role in cellular responses to DNA interstrand crosslinks. The precise role of FA proteins in ICL repair is not well understood, however, and is a highly active area of investigation.

### **FA-BRCA PATHWAY: COMPONENTS, INTERACTIONS, AND REGULATION**

For the purpose of understanding what is presently known about how FA proteins function, and for identifying potential therapeutic targets, we will examine identified FA proteins and their interactions. This overview will focus predominantly on work in mammalian systems, because of the direct relevance to tumor therapy.

#### **FA Nuclear Core Complex**

Eight of the identified FA proteins, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCD3, FANCI, and FANCG, form a nuclear complex (the FA nuclear core complex) which is required for the monoubiquitination of FANCD2 and FANCI [73-75, 82, 83, 99-101]. In addition, two other proteins, FAAP24 and FAAP100, which associate with FANCM and with FANCB/FANCL, respectively, are also associated with the FA nuclear core complex [102, 103]. Both FAAP24 and FAAP100 are required for FANCD2 monoubiquitination, but no FA patients with biallelic mutation of these genes have been identified. This network, including the FA nuclear core complex, along with FANCD2 and FANCI, constitutes what we will refer to as the basic

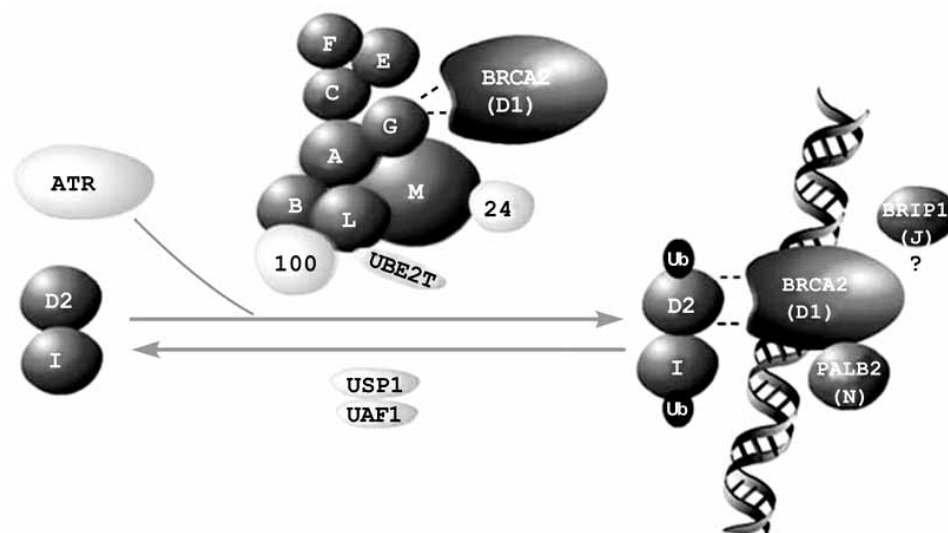
Fanconi anemia (FA) pathway. Three other FA proteins, all of which have some relationship to breast cancer [104-106], BRCA2/FANCD1, PALB2/FANCN, and BRIP1/FANCI, are not required for FANCD2 monoubiquitination [52, 85, 100, 107]. Along with components of the basic FA pathway, BRCA2, PALB2, and FANCI form an extended FA-BRCA pathway which is shown diagrammatically in Fig. (3).

Disruption of any of the known components of the FA nuclear core complex abrogates the monoubiquitination of FANCD2 and FANCI, either during S phase or in response to various DNA damaging agents (including ICLs) [73-75, 82, 83, 99, 100, 108]. Loss of certain FA nuclear core complex proteins can perturb function of the complex by destabilizing other core complex proteins or by disrupting their targeting to the nucleus. In particular, FANCM is required for the stability of FANCA and FANCG [75], and FANCA is required for the stability of FANCG [109]. FANCA and FANCE have nuclear localization signals [74, 76] and are required for the nuclear localization of FANCB and FANCC, respectively [74, 101, 110]. Interestingly, FANCE can bind to both FANCC and FANCD2, and may be an adaptor that bridges the FA nuclear core complex and its substrate, FANCD2 [101]. In accord with these results, there is biochemical evidence for possible subcomplexes formed by FA nuclear core complex proteins, including FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCI, FANCG, FANCE, FANCL, FANCM, FANCL, FANCL, and M [111]. Additionally, yeast two- and three-hybrid studies have mapped direct protein interactions within the FA nuclear core complex [112-114].

Four of the proteins within the FA nuclear core complex (FANCA, FANCB, FANCC, and FANCI) have identified structural or functional domains. Identified domains within FA proteins are outlined and discussed in detail in recent reviews [24, 115]. FANCA, FANCB, and FANCC have protein interaction domains which could potentially be targeted to disrupt FANCD2 monoubiquitination and increase sensitivity to ICLs. FANCG has 7 tetratricopeptide repeat motifs (TPR), which typically scaffold multi-protein complexes [116]. FANCL has a WD40 repeat domain, which mediates interactions with other proteins in the FA nuclear core complex [117]. FANCL also contains a plant homeodomain (PHD) at its C-terminus [73], a feature of RING-type E3 ubiquitin ligases.

FANCF interacts with both the FANCA/FANCG and FANCC/FANCE subcomplexes, and acts as an adaptor that is thereby required for the integrity of the FA nuclear core complex [118]. A structural determination has been completed for FANCF [119]. This study, along with analysis of FANCF mutants, has identified surfaces which mediate interactions with other FA nuclear core complex proteins [118, 119]. It is apparent, therefore, that there are numerous protein interactions within the FA nuclear core complex that are critical for resistance to agents which induce ICLs.

FANCM possesses a N-terminal DEAH-box helicase domain and a degenerate endonuclease domain at its C-terminus [75]. FANCM binds to Holliday junctions and to replication fork structures, and promotes branch migration in an ATPase-dependent manner [120, 121]. FANCM could also act as a motor that translocates the FA nuclear core complex on DNA [75, 122]. In agreement with this possibility, it has recently been demonstrated that FANCM is re-



**Fig. (3). Diagram of interrelationships of Fanconi anemia (FA) proteins and of the regulation of FANCD2 monoubiquitination.** At least eight of the FA proteins, FANCA, FANCB, FANCC, FANCE, FANCF, FANG, FANGL, and FANCM, form a complex which is predominantly nuclear and which is required for the monoubiquitination of the FA proteins, FANCD2 and FANCI, either during S phase or in response to DNA damage. This biochemical pathway is termed the FA pathway. FAAP24 and FAAP100 are associated with the FA nuclear core complex but have not been identified as FA genes (indicated by lighter colored symbols for these proteins in the figure). The ATR checkpoint kinase and USP1/UAF1 have also not been identified as FA proteins, but are critical for the regulation of FANCD2 monoubiquitination in response to DNA damage by an unknown mechanism and USP1/UAF1 deubiquitinates FANCD2. Monoubiquitinated FANCD2 is targeted to chromatin, where it interacts with the FA protein BRCA2/FANCD1. Monoubiquitinated FANCD2 organizes BRCA2/FANCD1 foci in response to DNA damage. PALB2/FANCN is a recently identified FA protein which is a partner of BRCA2/FANCD1 required for its stability and recruitment to chromatin. BRIP1/FANCI, like BRCA2/FANCD1 and PALB2/FANCN, is not required for FANCD2 monoubiquitination and is therefore proposed to function downstream of, or in association with, monoubiquitinated FANCD2. The functional relationship of BRIP1/FANCI to other FA proteins has not been determined.

quired for the association of the FA nuclear core complex with chromatin [123]. While the translocase and branch migration activities are ATPase-dependent, no helicase activity has been demonstrated for FANCM [121]. Inhibitors of FANCM ATPase activity could potentially disrupt processing of repair intermediates at stalled replication forks or the proper localization of the FA nuclear core complex to sites of DNA damage.

Monoubiquitination of FANCD2, as well as FANCI, appears to be a key function of the FA nuclear core complex [73-75, 82, 83, 99-101]. Monoubiquitination is a stable protein modification involved in cell signaling [124, 125]. The best characterized function of monoubiquitination is in regulation of protein trafficking between membrane compartments [124]. Monoubiquitin-binding motifs, such as the UBA (ubiquitin-associated) and CUE (motif in Cue1) domains mediate an interaction of "receptor" proteins with the monoubiquitinated protein in the endocytic process [125]. The importance of monoubiquitination of nuclear proteins in the DNA damage response has been described more recently. Monoubiquitination of PCNA [126], as well as the FA protein, FANCD2 [99], is critical in cellular responses to DNA damage. Monoubiquitination of PCNA mediates polymerase switching from replicative to TLS polymerases when DNA damage is encountered [127].

The system for ubiquitination involves an E1 enzyme, which activates ubiquitin by ATP hydrolysis and transfers it to an E2 ubiquitin conjugating enzyme. The E3 ligase mediates the transfer of activated ubiquitin to the substrate [128].

FANCL and its PHD domain can auto-ubiquitinate *in vitro* [73], suggesting that FANCL is the E3 component of the FA nuclear core complex which catalyses FANCD2 monoubiquitination. Importantly, a small molecule inhibitor of the MDM2 E3 ubiquitin ligase has been described [129] and assays are available for screening for inhibitors of E3 ligases [130]. This raises the possibility of increasing the sensitivity of cancer cells to ICLs by chemical inhibition of FANCL [131]. A potential FANCD2-E2 ligase, UBE2T, has also been identified [132].

It appears that there is also a mechanism which regulates the FA pathway by deubiquitinating FANCD2 (Fig. 3). An enzyme, USP1, has been identified which physically associates with, and which is required for, FANCD2 monoubiquitination [133]. A partner of USP1, UAF1, has recently been identified and this complex deubiquitinates FANCD2 *in vitro* [134]. Thus, USP1 may be involved in recycling monoubiquitinated FANCD2 to its inactive state. The potential importance of this step is suggested by the observation that USP1-deficient cells are sensitive to cisplatin and MMC [135].

The FA nuclear core complex may have other functions, in addition to its role in mediating FANCD2 and FANCI monoubiquitination. Interactions with non-FA proteins are suggestive of possible additional functions of FA proteins and may yield insight into how FA proteins are regulated (Table 1). For example, both FANCC and FANCG interact in the cytoplasm with enzymes involved in the detoxification of reactive oxygen species. FANCC interacts with cyto-

**Table 1. Interactions of FA Proteins with Non-FA Proteins**

FA Protein	Interacting Protein	Function	Interaction		Ref.
			IP	IF	
FANCA	BRCA1	DNA damage responses	x		[142]
	BRG1	Chromatin remodeling	x		[144]
	DAXX	Transcriptional regulation	x		[146]
	I $\kappa$ B $\gamma$	Transcriptional regulation	x		[146]
	Ran	Transporter	x	x	[146]
	SNX5	Transporter	x		[146]
FANCC	Cyt. P450 reductase	Anti-oxidant	x		[136]
	GST P1-1	Anti-oxidant	x		[137]
	Cdc2	Cell cycle control	x		[98]
	FAZF	Transcriptional regulation	x	x	[140]
FANCD1 (BRCA2)	BRCA1	DNA damage responses	x	x	[173]
	DSS1	DNA damage responses	x		[172]
	RAD51	DNA damage responses	x	x	[174]
	RPA	DNA damage responses	x		[171]
FANCD2	ATR	DNA damage responses		x	[147]
	BLM	DNA damage responses	x	x	[148, 150]
	BRCA1	DNA damage responses	x	x	[99, 108]
	NBS1	DNA damage responses	x	x	[44]
	PCNA	DNA damage responses		x	[153, 195]
	RAD51	DNA damage responses		x	[108]
	RPA	DNA damage responses		x	[147]
FANCG	Cyt. P450 reductase 2E1	Anti-oxidant	x		[138]
	Peroxiredoxin-3	Anti-oxidant	x	x	[139]
	XRCC3	DNA damage responses	x		[141]
FANCI (BRIP1)	BRCA1	DNA damage responses	x	x	[179, 182]
	RPA	DNA damage responses	x	x	[233]

chrome P450 reductase and glutathione S-transferase P1-1 [136, 137], and FANCG interacts with cytochrome P450 2E1 and the mitochondrial peroxidase, peroxiredoxin-3 [138, 139]. It has also been reported that FANCC associates with Cdc2 kinase [98], which regulates cell cycle progression, and with the FAZF protein [140], which is involved in transcriptional regulation. And FANCG binds to the RAD51 paralogue, XRCC3 [141], which is involved in HR [58].

Additionally, it has been reported that FANCA binds to the tumor suppressor BRCA1 [142], which is involved in DNA damage signaling (reviewed in [143]). FANCA has also been reported to associate with BRG1 [144], which has a role in chromatin remodeling. It has also been reported that the FA nuclear core complex is associated with the BLM tumor suppressor, the BLM partner topoisomerase III, and the single-strand DNA binding complex RPA [145]. These interactions are not listed in Table 1, since it is unknown whether BLM, topo III, or RPA directly interact with any of the FA nuclear core complex proteins.

A comprehensive yeast 2-hybrid study which used either of three different proteins of the FA nuclear core complex, FANCA, FANCC, and FANCG, as bait identified 69 potentially novel interactions [146]. Only a small subset of these potential interactions, including the interactions of DAXX, I $\kappa$ B $\gamma$ , Ran, and SNX5 with FANCA, were confirmed by co-immunoprecipitation or co-localization studies, however [146]. Major groups of proteins that were identified include those involved in transcriptional regulation, oxidative metabolism, cell signaling, cellular and nuclear transport, and DNA repair [146].

#### **FANCD2, FANCI, BRCA2/FANCD1, PALB2/FANCN, and BRIP1/FANCI**

Monoubiquitinated FANCD2 assembles into nuclear foci, where it colocalizes with various DNA damage response proteins, including BRCA1, BRCA2, RAD51, NBS1, BLM, ATR, and RPA2, following exposure to DNA damaging agents [44, 99, 108, 147-150] (Table 1). FANCD2 also

colocalizes with BRCA1 and RAD51 during S phase, in the absence of exogenous DNA damage [99, 108]. This result suggests the possibility of a closer functional relationship of FANCD2 with BRCA1 and RAD51, potentially in HR given the known functions of BRCA1 and RAD51 in this process [151, 152]. Co-immunoprecipitation of FANCD2 with BRCA1, BRCA2/FANCD1, and NBS1 has been reported [44, 99, 149, 153] (see Table 1). Although these interactions could be indirect, these co-immunoprecipitation results raise the possibility of a functional relationship of FANCD2 with BRCA1, BRCA2, and NBS1. The identification of additional binding partners of FANCD2 should yield important insights into the function of monoubiquitinated FANCD2 and may identify potential therapeutic targets.

FANCD2 monoubiquitination is critical for the assembly of FANCD2 into nuclear foci, for targeting FANCD2 to chromatin, and for resistance to DNA interstrand crosslinkers. This is demonstrated most clearly by experiments in which a non-ubiquitinable mutant of FANCD2, modified at the site of ubiquitin conjugation (K561 in human and K563 in chicken), is expressed in cells lacking endogenous FANCD2 protein [99, 149, 154, 155]. Importantly, the phenotype of these cells matches those observed in cells deficient for various components of the FA nuclear core complex [99, 149], reflecting the essential function of the FA nuclear core complex in mediating FANCD2 monoubiquitination. It should also be noted that examination of available FA-D2 cell lines demonstrates that hypomorphic mutations in the FANCD2 gene result in strongly decreased levels of FANCD2 that can be monoubiquitinated [156]. This supports the conclusion that the cellular capacity to monoubiquitinate FANCD2 is critical for the collective function of FA proteins.

It has been proposed that a “receptor” for monoubiquitinated FANCD2 might target FANCD2 to chromatin and to nuclear foci [115, 154], paralleling the function of monoubiquitinated proteins in endocytosis [125]. Such a receptor remains to be identified, however.

The ATR checkpoint kinase appears to couple FANCD2 monoubiquitination to DNA damage response signaling mechanisms (Fig. 3) [147, 157]. ATR and RPA, which is required for ATR activation [158], are specifically required for FANCD2 monoubiquitination in response to DNA damage [147, 157]. ATR may directly phosphorylate FANCD2 in response to DNA damage induced by ICLs [159], or by exposure to either UV or ionizing radiation [160]. Importantly, chemical inhibition of ATR activity suppresses FANCD2 monoubiquitination [147]. Phosphorylation of the histone protein H2AX may also be involved in ATR-dependent regulation of FANCD2 monoubiquitination [161]. Thus, inhibition of ATR activity could sensitize cancer cells to agents which induce ICLs. The ATM checkpoint kinase also phosphorylates FANCD2 [160, 162], but is not required for FANCD2 monoubiquitination.

FANCI displays limited sequence homology to FANCD2 and is monoubiquitinated in a similar position within the protein [82, 83]. FANCI forms DNA damage-induced foci that colocalize with FANCD2 foci [82]. The assembly of FANCI foci requires monoubiquitination of FANCI. While FANCI and FANCD2 can form a protein complex, a major-

ity of the FANCD2 protein present in the cell is not associated with the complex [82, 83]. The functional importance of the FANCD2-FANCI complex is currently unknown.

Interestingly, FANCI and FANCD2 are required for the monoubiquitination of the other by an unknown mechanism [82, 83]. Importantly, the non-ubiquitinable FANCI-K523 mutant largely restores MMC resistance in FANCI-deficient cells [82]. This is in contrast to the absolute requirement for FANCD2 monoubiquitination for resistance to MMC [99, 162]. Like FANCD2, FANCI appears to be phosphorylated by ATM and/or ATR in response to DNA damage [82]. The functional significance of these modifications has not been determined, however.

BRCA2/FANCD1, PALB2/FANCN, and BRIP1/FANCI are not required for FANCD2 monoubiquitination [52, 85, 100, 107]. The interaction of these proteins with monoubiquitinated FANCD2, or with other FA proteins, is currently a subject of intense research interest and offers the potential for important insights into the function of FA proteins. A linear pathway in which FANCD2, activated by monoubiquitination, regulates or organizes “downstream” FA proteins, such as BRCA2/FANCD1, was initially proposed [163]. FANCD2 interacts directly with BRCA2 [153], and monoubiquitinated FANCD2 interacts with BRCA2/FANCD1 in chromatin [149]. Further, monoubiquitinated FANCD2 organizes BRCA2 nuclear foci in response to DNA damage induced by ionizing radiation [149].

The hypothesis that BRCA2/FANCD1 functions exclusively at the end of the FA pathway, in a manner that is dependent upon monoubiquitinated FANCD2, has been challenged by more recent studies, however [141, 149, 164-166]. These experiments have suggested that BRCA2/FANCD1 interacts with components of the FA nuclear core complex, including FANCE and FANCG. A direct interaction between FANCG and BRCA2 has been demonstrated by yeast 2-hybrid studies [164], while it is unknown whether FANCE interacts with BRCA2 directly [149]. Further, FA-D1 cells, which have biallelic BRCA2 mutations [52], display important phenotypic differences from cells of other FA complementation groups.

FA-D1 patients display many of the clinical and cellular phenotypes that are characteristic of FA patients, including growth retardation, skeletal anomalies, microcephaly, and café-au-lait spots [167]. This suggests the functional link of BRCA2 to other FA proteins. But other phenotypes, most notably a markedly earlier onset of leukemia [167, 168], may be reflective of the direct role of BRCA2 in DNA repair. Alternatively, the more severe phenotype of FA-D1 patients could result from additional functions BRCA2 may have, which are independent of other FA proteins [90, 166].

Another indication that BRCA2 may have functions which are distinct from other FA proteins comes from the finding that cells from most FA complementation groups, in contrast to FA-D1 cells which are deficient for BRCA2, have a modest deficiency in HR [165, 169]. One possibility is that FA proteins, other than BRCA2/FANCD1 and PALB2/FANCN, mediate HR but do not have a direct mechanistic role in this process. Potential roles for FA proteins in DNA repair will be discussed in the next section.

BRCA2 physically interacts with various DNA damage response proteins, including BRCA1, DSS1, and RPA. BRCA2 is also a component of a complex with E3 ubiquitin ligase activity that also contains BRCA2, BRCC36 and BRCC45 (Table 1) [170-174].

PALB2/FANCD1 has recently been identified as a partner of BRCA2 which regulates its stability, and its interaction with chromatin and the nuclear matrix in response to DNA damage [175]. FA-N patients, like FA-D1 patients, have a more severe and tumor-prone phenotype than FA patients from other complementation groups [84, 85], indicative of the important functional relationship between PALB2 and BRCA2. The relationship of FANCD2 to PALB2 is unknown, however. But while BRCA2 and PALB2 are clearly required for the assembly of RAD51 DNA damage foci, other FA proteins, including FANCD1/BRIP1, have at most minor roles in regulating the assembly of RAD51 foci [149, 165, 176-178].

BRIP1, also known as BACH1, was first identified as a BRCA1-interacting protein [179]. Subsequently, BRIP1 was identified as the FA gene FANCD1 [71, 72, 97]. Interestingly, BRCA1 has not been identified as a FA gene [180]. Physical interaction of BRIP1/FANCD1 with FA proteins other than BRCA2 has not been found [97, 181] and the functional relationship of BRIP1 to other FA proteins has not been determined. Human BRIP1 interacts with the C-terminus of BRCA1 *via* phosphorylation of BRIP1 at S990 [181, 182]. BRIP1 phosphorylation at the S990 site is cell cycle regulated and is a potential therapeutic target [182]. While it has been reported that BRIP1 phosphorylation at S990 is required for G2 checkpoint function [182], other FA cells have a functional G2 checkpoint [96, 162]. The N-terminus of BRIP1 contains DEAH helicase boxes [179], and BRIP1 is a functional DNA helicase *in vitro* [183]. Helicase inhibitors have been described [184]. Since BRIP1 helicase activity is required for cellular resistance to MMC [185], a specific inhibitor of BRIP1/FANCD1 could potentially sensitize cells to ICLs.

A protein complex containing all of the identified FA proteins together has not been reported [25], but remains a possibility. The existence of such a complex might suggest that the FA nuclear core complex has a closer functional relationship to other FA proteins, including FANCD2, FANCD1, BRCA2, PALB2, and BRIP1, than if the FA nuclear core complex does not physically associate with these proteins.

### **FANCONI ANEMIA PROTEINS AND THEIR POSSIBLE ROLES IN CELLULAR RESPONSES TO DNA DAMAGE**

We suggest that the characteristic cellular phenotype observed in cells from FA patients likely reflects a repair defect, rather than a checkpoint defect. For example, defective S phase checkpoint function has been reported for cells from some, but not all, FA complementation groups. FA-D1 and FA-D2 cells from human patients, which are deficient for BRCA2/FANCD1 and FANCD2, respectively, display radio-resistant DNA synthesis (RDS) upon exposure to ionizing radiation [90, 149, 162]. In contrast, a RDS phenotype is

not observed in human FA-C cells, which lack FANCD2 [162]. Surprisingly, mouse cells lacking FANCD2 are hypersensitive to ICLs but do not display the RDS phenotype [186]. Thus, it appears that a S phase checkpoint defect is not central to the FA cellular phenotype.

FA cells, of most complementation groups, also have normal G2 checkpoint function. FANCD2-deficient cells display normal DNA damage-induced induction of p53 and the checkpoint inhibitor caffeine inhibits MMC-induced G2-M accumulation in these cells [88]. Further, FA-C cells and their counterparts, corrected by exogenous expression of FANCD2, display equivalent G2-M accumulation after exposure to equitoxic doses of mitomycin C, as calculated by levels of cytogenetic damage [96].

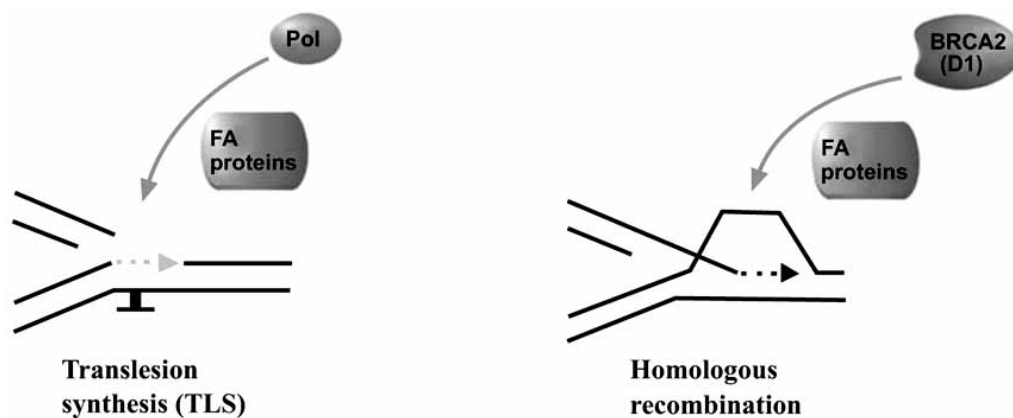
Although FA proteins do not appear to have an integral role in cell cycle checkpoint function, evidence exists for a role in DNA repair [150, 169, 178, 187-189]. Thus, the question arises, what is the role of FA proteins in DNA repair? As described above, the repair of ICLs is a multi-step process that involves various DNA repair pathways (see Fig. 2). It has been proposed that FA proteins may coordinate the various steps involved in the repair of ICLs [26], and evidence suggests that FA proteins have a critical role in this process downstream of incision and the generation of a DNA double-strand break [31, 34].

Results obtained with mammalian cells and chicken DT40 cells suggest that the FA pathway may cooperate with TLS and HR in ICL repair [187, 190, 191]. FA proteins could promote TLS and HR by recruiting proteins such as TLS polymerases or BRCA2 to specific DNA repair intermediates (Fig. 4), or by processing of these intermediates to generate substrates for TLS or HR.

Potentially related to a role in promoting HR, it has been suggested that FA proteins either have a function in preventing the collapse of, or in restarting, stalled replication forks [18, 192]. HR is required to restart stalled replication forks [54] and, consistent with its role in HR [193], it has been reported that BRCA2 is required to stabilize stalled replication forks [194]. Additionally, FANCD2, and perhaps the FA nuclear core complex, appear to be recruited to sites of DNA replication stalled by DNA damage [153, 195, 196]. FANCD2 is not a constitutive component of the replication fork, however. Also consistent with a possible role in stabilizing stalled replication forks, the FA pathway suppresses chromosome breakage at common fragile sites in response to replication stress [195].

### **POTENTIAL STRATEGIES FOR THE SENSITIZATION OF MALIGNANT CELLS TO ICLs**

Many tumor cells have increased DNA repair capacity as a mechanism of acquired drug resistance, limiting the efficacy of therapeutic agents which induce DNA damage (reviewed in [3, 197]). A recent strategy combines DNA damaging agents with inhibitors of the specific repair pathways which are activated (reviewed in [1, 3, 4, 197]). Among the approaches which have been proposed for therapeutic applications are antisense or RNAi (RNA Interference)-mediated suppression of target proteins [198-200], and the development of small molecules which inhibit the activity of target



**Fig. (4).** Diagram of the possible functions of Fanconi anemia (FA) proteins in promoting translesion synthesis (TLS) and homologous recombination (HR). FA proteins could recruit or organize DNA damage response proteins, such as bypass polymerases (left) or BRCA2 (right) and thereby promote TLS or HR, respectively.

proteins [201-204]. Isogenic human cancer cell lines generated by targeted disruption of either FANCC or FANCG are a helpful preclinical model [205].

Recent reports suggest that suppression or inhibition of FA proteins, or other proteins which function in ICL repair, can sensitize tumor cells to ICL-inducing agents. As an example, siRNA (small interfering RNA)-mediated suppression of the nucleotide excision repair (NER) protein ERCC1 sensitizes ovarian cancer cell lines to cisplatin [206]. Further, siRNA-mediated suppression of FANCF sensitizes multiple myeloma cells to the DNA interstrand crosslinker, melphalan [207]. Expression of a dominant-negative form of FANCA using adenoviruses also sensitizes tumor cells to DNA interstrand crosslinkers [208]. Importantly, suppression of any component of the FA nuclear core complex, by antisense or RNAi-mediated approaches, could potentially result in deficient FANCD2 monoubiquitination and sensitize tumors to agents which induce ICLs.

A screen of siRNA libraries for sensitization of tumor cell lines to cisplatin [209], has established the potential of this approach to identify siRNAs with therapeutic value. Further, proteins were identified which can potentially be targeted by small molecules to sensitize tumor cells to ICLs, including BRCA1, BRCA2, BARD1, RAD51, ATR and CHK1 [209].

The potential of small molecule inhibitors to sensitize tumor cells to DNA interstrand crosslinking agents is demonstrated by potentiation of the effects of cisplatin by protein kinase inhibitors, including TOR and MEK kinase inhibitors, and a non-specific Chk1 kinase inhibitor [201-203]. Further, inhibitors of the ATR checkpoint kinase should sensitize cells to ICLs since ATR regulates FANCD2 monoubiquitination [147, 157].

A cell-based screening assay for small molecule inhibitors of the FA pathway has been described [204]. A library was screened for inhibition of the assembly of FANCD2 foci induced by ionizing radiation in cells expressing EGFP-FANCD2. From this screen, four inhibitors of the assembly of FANCD2 foci have been described, including three protein kinase inhibitors. Among the protein kinase inhibitors was wortmannin, which is consistent with its inhibition of

ATR-dependent DNA damage-induced FANCD2 monoubiquitination [147]. The other two compounds were the broad-specificity inhibitors H-9 and alsterpaullone [204]. H-9 inhibits protein kinase A, protein kinase C, protein kinase G, calcium/calmodulin-dependent protein kinase, and myosin light chain kinase [210, 211]. Alsterpaullone inhibits the cyclin-dependent kinases CDK1, CDK2, and CDK5, as well as glycogen synthase kinase-3B [212, 213]. Since FANCD2 monoubiquitination and assembly into nuclear foci appears to require cell cycle progression through S phase [34, 108, 192], inhibition of the assembly of FANCD2 foci by these latter two inhibitors could possibly be attributable to cell cycle arrest, however. The fourth inhibitor identified was curcumin (diferuloylmethane) [204], a natural product with previously demonstrated anti-inflammatory and anti-neoplastic activities [214]. Importantly, curcumin also inhibits FANCD2 monoubiquitination and sensitizes breast and ovarian tumor cell lines to cisplatin, but does not itself induce cell cycle arrest [204]. Small molecule inhibitors identified from such screens might sensitize tumor cells to a broad spectrum of the various drugs which induce ICLs.

It has recently been demonstrated that inhibitors of poly(ADP-ribose) polymerase (PARP), which is involved in base excision repair (BER), are highly cytotoxic to tumor cells deficient for various HR-related proteins. Cells deficient for BRCA1, BRCA2, FANCA, FANCC, FANCD2, RAD51, RAD54, CHK1, and ATR, all of which are involved in ICL repair, are sensitive to PARP inhibitors [215-217]. Thus, PARP inhibitors may be highly effective in personalized therapies based upon deficiencies in HR-related genes.

There are potential challenges, however, to the implementation of the strategies discussed above. For example, pathways that function in the repair of ICLs may be inhibited both in tumor cells and normal cells in the body. Success with such approaches may require methods to specifically deliver RNAi or small molecule inhibitors to the tumor. Another potential difficulty is the finding that tumor cells frequently inactivate apoptotic mechanisms that would normally eliminate cells that have accumulated extensive DNA damage following treatment with chemotherapeutic agents (reviewed in [218]).

## SOMATIC MUTATION AND SILENCING OF FA GENES IN CANCER IN NON-FA PATIENTS

Effective cancer therapy should selectively kill malignant cells and not non-malignant cells. Recent advances have identified malignancies that show acquired inactivation of FA proteins (reviewed in [219, 220]). Since inactivation of FA proteins is associated with hypersensitivity to ICLs [19, 22-25], such tumors should be selectively killed by agents which induce ICLs. Assays to identify tumors in which FA proteins are inactivated should in the future lead to personalized therapies [6]. Inactivation of FA proteins could be determined by biochemical assays, such as measurements of the capacity for FANCD2 monoubiquitination, by the use of cell-based assays, such as measurements of sensitivity, or by detection of sequence alterations in FA genes.

Mutation of BRCA2/FANCD1, or of BRCA1, and subsequent loss of heterozygosity (LOH), is of critical importance to the onset of familial breast cancer, as well as ovarian, pancreatic, and prostatic cancer [221]. Large scale studies have recently identified PALB2/FANCN and BRIP1/FANCI as low penetrance inherited breast cancer genes [104, 106]. A study of FA heterozygotes has indicated that FANCC may also be a breast cancer gene [222]. It has been reported that women with BRCA1 or BRCA2-associated ovarian cancer showed improved survival following treatments that included carboplatin, relative to sporadic cases [223]. This study suggests the potential for personalized treatment of tumors with specific inactivation of the FA pathway with agents that induce ICLs.

Studies have suggested that mutation of FANCC, in addition to mutation of BRCA2/FANCD1, may be involved in sporadic pancreatic cancer [220]. Acquired somatic mutation of BRCA2/FANCD1, and of FANCA, has also been associated with sporadic cases of acute myeloid leukemia (AML) [220, 224-226]. For example, one study found heterozygous deletions of FANCA in 4/101 cases of sporadic AML [225] and another uncovered point mutations of FANCA in 5/79 cases of AML [226].

In contrast to FANCA, FANCI, FANCD1, FANCF, and FANCG, which can be inactivated by mutation in sporadic cancer, as described above, epigenetic inactivation of FANCF is found in a variety of tumors [219]. For example, hypermethylation of the CpG island in the promoter for FANCF was found in 2/25 ovarian cancer cell lines [227]. In this study, hypermethylation of the FANCF promoter was associated with a deficiency in FANCD2 monoubiquitination and with sensitivity to the ICL-inducing agent cisplatin [227]. FANCF was also hypermethylated in 4/19 primary ovarian tumors [227]. Additionally, an increased incidence of FANCF hypermethylation was found in primary cervical cancers or cervical cancer cell lines [228], in squamous cell carcinomas of the head/neck [229], in non-small-cell lung carcinomas [229], in bladder cancer [230], and in an AML-derived cell line [231]. The importance of hypermethylation of the FANCF gene to therapeutic outcome is underscored by the association of an unmethylated FANCF gene with acquired resistance of ovarian cancers to cisplatin [227]. Hypermethylation of the promoter for an additional FA gene, PALB2/FANCN, has recently been reported in a subset of breast and ovarian tumors [232].

## CONCLUDING REMARKS

Numerous pathways cooperate in the repair of ICLs, including checkpoint kinases, nucleotide excision repair (NER), translesion synthesis (TLS), homologous recombination (HR), and Fanconi anemia (FA) proteins. This has generated a large number of potential targets for therapeutic intervention, including proteins which have catalytic activities and which might therefore be susceptible to chemical inhibition. One strategy for intervention is sensitization of malignant cells to ICLs, either by chemical inhibition or RNAi-mediated suppression of proteins which function in the repair of such lesions. An increased understanding of the pathways which function in ICL repair, including the FA pathway, has led to functional screens which can be utilized for identifying effective suppressors of ICL repair. Importantly, immunoblotting or immunofluorescence microscopy for the function of the FA pathway, as measured by FANCD2 monoubiquitination or foci formation, respectively, are potential screens for novel therapeutic agents.

Recent progress in the identification of FA genes, and in understanding their function, may present novel therapeutic targets related to the repair of ICLs. Critically, at least eight of the FA proteins form a nuclear complex which is required for the monoubiquitination of FANCD2. Inhibition of the FANCD2 ubiquitin ligase activity or activation of FANCD2 deubiquitination may represent potential therapeutic strategies. Additionally, the ATPase activities of FANCM and FANCI may also be potential targets for the modulation of ICL repair. And it might be possible to sensitize cancer cells to ICL-inducing agents by suppressing or inhibiting the function of BRCA2 in DNA repair. FA proteins interact with many of the other pathways involved in ICL repair and further identification of such interactions may provide additional therapeutic targets.

Finally, the continued identification of components of the network involved in ICL repair is important for the potential development of personalized therapies. Loss of BRCA1 or BRCA2 function, or hypermethylation of FANCF, are examples in which mutation or epigenetic suppression of proteins involved in ICL repair may be associated with the sensitization of particular tumors to ICLs. Such personalized therapies will be facilitated by a better characterization of how ICL repair pathways are inactivated in various malignant diseases and will require the development of efficient assays to detect inactivation of specific ICL repair proteins in tumors.

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## ABBREVIATIONS

AML = Acute Myeloid Leukemia  
ATP = Adenosine-5'-triphosphate

CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-urea  
 CUE = Motif found in yeast Cue1 protein  
 DEB = Diepoxybutane  
 DNA = Deoxyribonucleic Acid  
 FA = Fanconi Anemia  
 G2-M = Gap Phase 2/Mitosis  
 HR = Homologous Recombination  
 ICL = Interstrand Crosslink  
 LOH = Loss of Heterozygosity  
 MMC = Mitomycin C  
 MMR = Mismatch Repair  
 MRN = MRE11-RAD50-NBS1 complex  
 NER = Nucleotide Excision Repair  
 PARP = Poly(ADP-Ribose) Polymerase  
 PHD = Plant Homeodomain  
 RNAi = RNA Interference  
 RDS = Radioresistant DNA Synthesis  
 siRNA = Small Interfering RNA  
 TLS = Translesion Synthesis  
 TPR = Tetratricopeptide repeat motifs  
 UBA = Ubiquitin-associated  
 UV = Ultraviolet light

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