

REVIEW ARTICLE

The Fanconi anemia pathway and ICL repair: implications for cancer therapy

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Abstract

Fanconi anemia (FA) is an inherited disease caused by mutations in at least 13 genes and characterized by genomic instability. In addition to displaying strikingly heterogeneous clinical phenotypes, FA patients are exquisitely sensitive to treatments with crosslinking agents that create interstrand crosslinks (ICL). In contrast to bacteria and yeast, in which ICLs are repaired through replication-dependent and -independent mechanisms, it is thought that ICLs are repaired primarily during DNA replication in vertebrates. However, recent data indicate that replication-independent ICL repair also operates in vertebrates. While the precise role of the FA pathway in ICL repair remains elusive, increasing evidence suggests that FA proteins function at different steps in the sensing, recognition and processing of ICLs, as well as in signaling from these very toxic lesions, which can be generated by a wide variety of cancer chemotherapeutic drugs. Here, we discuss some of the recent findings that have shed light on the role of the FA pathway in ICL repair, with special emphasis on the implications of these findings for cancer therapy since disruption of FA genes have been associated with cancer predisposition.

Keywords: DNA repair; DNA replication; crosslink; checkpoint; Fanconi anemia

Introduction

Fanconi anemia (FA) is a rare inherited disease associated with genomic instability that has attracted the interest of a diverse audience, from scientists studying the mechanisms of DNA repair to oncologists treating a variety of sporadic cancers. FA patients are characterized by developmental abnormalities, progressive bone marrow failure, and a predisposition to cancer, especially leukemias and carcinomas. Despite the diverse clinical phenotypes of the disease, a hallmark of cells derived from FA patients is a severe cellular hypersensitivity to crosslinking agents such as mitomycin C (MMC), cisplatin (CDDP), and diepoxybutane (DEB) (reviewed in Niedernhofer *et al.*, 2005; Patel and Joenje, 2007; Wang, 2007; Moldovan and D'Andrea, 2009; Thompson and Hinz, 2009). This characteristic has led to a great deal of research implicating the FA

pathway in crosslink repair and the maintenance of genomic stability. Furthermore, the FA pathway has also been associated with cancer susceptibility, primarily breast tumors, and with sensitivity and resistance to chemotherapeutic agents. Therefore, a better understanding of the mechanisms and roles of the FA pathway will not only help patients suffering from this rare disease, but also have an impact on cancer patients in the general population.

The FA pathway

Fanconi anemia results from mutations in one of 13 FA genes: *Fanca*, *-b*, *-c*, *-d1*, *-d2*, *-e*, *-f*, *-g*, *-i*, *-j*, *-l*, *-m* and *-n* (Table 1). FA-associated mutations are autosomal recessive apart from *Fancl*, which is X-linked. The proteins encoded by these genes make up the FA

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(Received 29 April 2010; revised 01 June 2010; accepted 14 June 2010)

ISSN 1040-9238 print/ISSN 1549-7798 online © 2010 Informa Healthcare USA, Inc.
DOI: 10.3109/10409238.2010.502166

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Table 1. Fanconi proteins, functions and connections to cancer.

FA protein	Sub-complex	Role	Connection to cancer
FANCA	Core complex	D2/I ubiquitylation, phosphorylated upon damage (ATR)	Inactivated by promoter methylation
FANCB	Core complex	D2/I ubiquitylation	
FANCC	Core complex	D2/I ubiquitylation	Inactivated by promoter methylation in AML and ALL
FANCE	Core complex	D2/I ubiquitylation, phosphorylated upon damage (CHK1)	
FANCF	Core complex	D2/I ubiquitylation	Inactivated (methylation) in ovarian, lymphoid, bladder, cervical and lung cancer
FANCG	Core complex	D2/I ubiquitylation, phosphorylated in M	
FANCL	Core complex	E3 ligase for D2/I complex	Altered expression in splice variants
FANCM	Core complex localization to DNA	Helicase/translocase phosphorylated in M	
FANCD2	Ubiquitylated complex	Ub and phosphorylated upon damage (ATM, ATR, CHK1)	
FANCI	Ubiquitylated complex	Ub and phosphorylated upon damage (ATR)	
FANCD1/BRCA2	Downstream foci	HR component	Breast, ovarian, prostate, pancreas cancer susceptibility, cisplatin sensitivity
FANCI/BACH1	Downstream foci	Helicase/translocase	Breast cancer susceptibility
FANCN/PALB2	Downstream foci	FANCD1 binding	Breast cancer susceptibility. Inactivated by promoter methylation

pathway. The FA pathway can be separated into three groups: the FA core complex, the FANCD2/FANCI (ID) complex, and FA proteins acting downstream. For more extensive reviews of the FA pathway, see Kennedy and D'Andrea (2005), Levitus *et al.* (2006), Mathew (2006) and Taniguchi and D'Andrea (2006). The FA core complex consists of FANCA, -B, -C, -E, -F, -G, -L, and -M, and accessory proteins FAAP24 and FAAP100. The downstream FA proteins consist of FANCD1, FANCI, and FANCN, which are also known as BRCA2, BRIP1/BACH1, and PALB2, respectively. FANCD1/BRCA2 is a major breast and ovarian cancer susceptibility gene and plays an essential role in homology-directed repair (HDR) (Godthelp *et al.*, 2006a; 2006b). FANCI interacts directly with BRCA1 and is a member of the DEAH and XPD helicase families (Cantor *et al.*, 2001; White, 2009). FANCN interacts with FANCD1/BRCA2 and is required for its recombination and checkpoint functions (Xia *et al.*, 2006a; Xia *et al.*, 2006b). This striking association between the FA pathway and breast cancer susceptibility appears to be restricted to this subset of FA genes (Garcia *et al.*, 2009). The FA pathway is a unique example in which mutations from a large epistatic group of genes are associated with the same disorder. Recently, a homozygous mutation in RAD51C, a RAD51 paralog, was identified in a family with multiple congenital abnormalities characteristic of FA (Vaz *et al.*, 2010). Based on the effect of RAD51C loss of function

on RAD51 foci formation, RAD51C appears to operate downstream of the ID complex.

Modifications of FA proteins: ubiquitylation

Central to the function and the regulation of the FA pathway is the ubiquitylation of the FANCD2/FANCI complex. The FA core complex monoubiquitylates FANCD2 and FANCI and activates the FA pathway (Garcia-Higuera *et al.*, 2001; Meetei *et al.*, 2003; Ciccia *et al.*, 2007; Dorsman *et al.*, 2007; Ling *et al.*, 2007; Sims *et al.*, 2007). While FANCL is the catalytic subunit (Meetei *et al.*, 2003), all 10 members of the FA core complex are required for its ubiquitin E3 ligase activity and UBE2T serves as the ubiquitin E2 ligase (Machida *et al.*, 2006; Alpi *et al.*, 2007; 2008). FANCM, however, can be distinguished from the other components of the core complex since FANCD2 is still partially ubiquitylated and the core complex remains intact following FANCM inactivation (Kim *et al.*, 2008). This has led to the proposal that FANCM-FAAP24 is a subunit targeting the core complex to DNA (Ciccia *et al.*, 2007; Ali *et al.*, 2009). In addition, FANCM and FAAP24 have been shown to interact with HCLK2 independently of the FA core complex and to function in ATR-mediated checkpoint signaling (Collis *et al.*, 2008). Another accessory protein, FAAP100, has been shown to interact in a stable complex with FANCL and FANCB and is essential

for FANCD2 monoubiquitylation and stability of the FA core complex (Ling *et al.*, 2007). Monoubiquitylation of FANCD2 and FANCI is critical for activation of the FA pathway as it is required for the formation and the localization of the FANCI/D2 complex to nuclear foci (Dorsman *et al.*, 2007; Sims *et al.*, 2007; Smogorzewska *et al.*, 2007; Wang, 2007). Functional grouping of FA proteins has been deduced from their roles in FANCD2/FANCI ubiquitylation (Figure 1). Components of the core complex are all required for ID modification and are therefore upstream. Inactivation of the downstream FA proteins does not affect ID ubiquitylation. The ID complex is formed by monoubiquitylated FANCD2 and FANCI whose monoubiquitylations are mutually dependent (Smogorzewska *et al.*, 2007). The ID complex is formed and localized to chromatin during the S-phase (Taniguchi *et al.*, 2002a) and in response to DNA damaging agents including MMC, ionizing radiation, ultraviolet light, and hydroxyurea (Garcia-Higuera *et al.*, 2001). On DNA, the ID complex forms foci with DNA repair proteins BRCA1 and RAD51, and FA downstream proteins (Garcia-Higuera *et al.*, 2001; Taniguchi *et al.*, 2002a; Wang *et al.*, 2004). Whereas ubiquitylation of FANCD2 at K561 is essential for the activation of the FA pathway, ubiquitylation of FANCI at K523 is not (Ishiai *et al.*, 2008).

The USP1/UAF1 complex deubiquitylates FANCD2 (Cohn *et al.*, 2007; Nijman *et al.*, 2005) and FANCI (Longerich *et al.*, 2009). Deubiquitylation of FANCD2 is required for ICL repair (Oestergaard *et al.*, 2007) and loss of USP1 is associated with ICL sensitivity (Kim *et al.*, 2009). Notably, inactivation of USP1 in mice recapitulates several FA phenotypes reminiscent of the knock-out of FA genes (Kim *et al.*, 2009). Proteasome inhibitors sensitize cells to DNA damage and a potential target for this sensitization is FA pathway activation (Jacquemont and Taniguchi, 2007). However, the specific protein target(s) of the proteasome has not been identified.

Modifications of FA proteins: phosphorylation

Several FA proteins are phosphorylated (Table 1; Figure 1). These modifications regulate the activity of the FA pathway following DNA damage and during the cell cycle. Whereas phosphorylation of FA proteins generally occurs in response to both DNA damage and replication stress, FANCA is specifically phosphorylated by ATR (ATM ataxia telangiectasia mutated) and Rad3-related) following DNA damage but not during DNA replication (Collins *et al.*, 2009). FANCM is phosphorylated in response to DNA replication and DNA damage and this phosphorylation is regulated by the PIKKs ATM and ATR (Kim *et al.*,

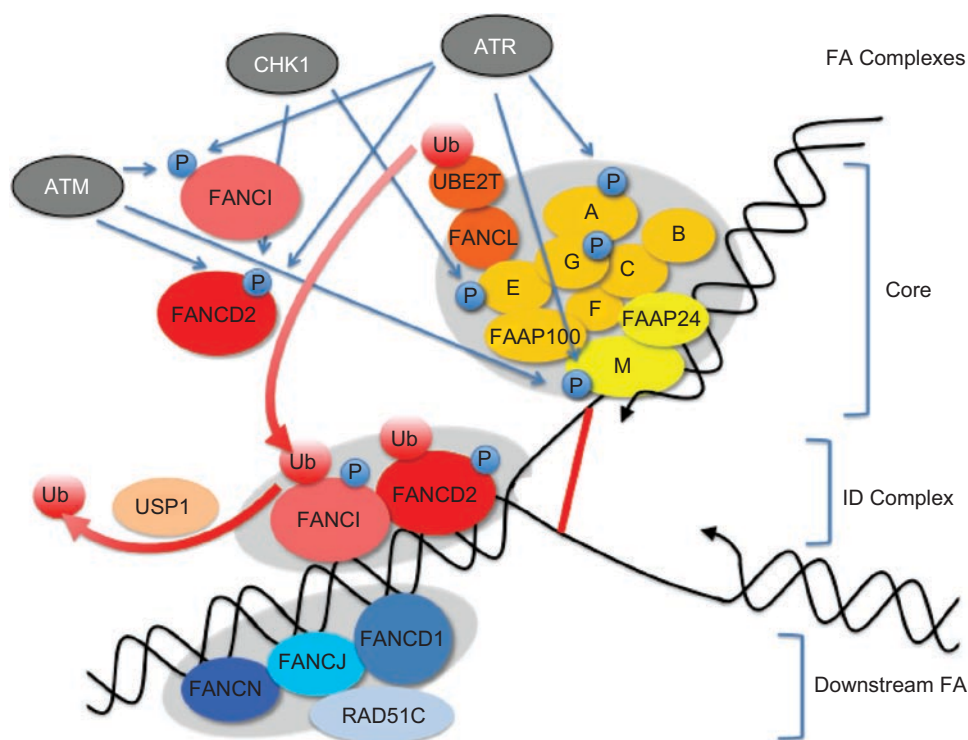


Figure 1. FA pathway: subcomplexes, proteins and post-translational modifications. Hypothetical localization of the different FA subcomplexes at a replication fork encountering an interstrand crosslink (red line). The core complex together with the E2 ligase UBE2T mono-ubiquitylate FANCI and FANCD2 (ID complex), thus triggering its association to damaged chromatin. Several FA proteins are phosphorylated in response to DNA damage by the checkpoint kinases ATM, ATR and CHK1. In addition, FANCM and FANCG are phosphorylated in mitosis (See text for details).

2008; Sobeck *et al.*, 2009). The consequences of the DNA damage-dependent phosphorylation of FANCM are not known. FANCD2 is phosphorylated at multiple sites following DNA damage. Phosphorylation by ATR is critical for checkpoint signaling during S-phase (Andreassen *et al.*, 2004) and for monoubiquitylation of FANCD2 (Ho *et al.*, 2006). FANCD2 is also phosphorylated by ATM; however, this modification is independent of FANCD2 monoubiquitylation (Taniguchi *et al.*, 2002b). FANCD2 and FANCE are phosphorylated by CHK1 and these modifications play a role in resistance to DNA crosslinking agents (Wang *et al.*, 2007; Zhi *et al.*, 2009). Finally, FANCG and FANCM are hyper-phosphorylated during mitosis (Kee *et al.*, 2009; Mi *et al.*, 2004). While the role of these cell cycle-dependent phosphorylations is not known, they could reflect a potential role for the FA pathway in mitosis. Notably, a genome-wide siRNA screen for genes that regulate mitosis progression showed that FANCC and FANCE downregulation results in mitosis delay (Neumann *et al.*, 2010). Furthermore, FANCD2 localizes to ultrafine chromosome bridges during anaphase, where it co-localizes with BLM (Neumann *et al.*, 2010), suggesting a possible role for FANCD2 in preventing aneuploidy (Naim and Rosselli, 2009).

Crosslinking agents and DNA interstrand crosslinks

ICLs are particularly toxic DNA lesions because they involve both strands of DNA, blocking the essential processes that require translocation along the DNA; namely, DNA replication and transcription. In addition to this physical constraint on DNA, ICLs require repair of damage on both strands of the DNA. Comparative studies ranking *in vitro* and *in vivo* genotoxicity of large sets of compounds have ranked crosslinking agents among

the most toxic (Lohman, 1999). DNA crosslinking agents cause gross chromosomal aberrations including chromosome loss, deletions, and breaks, and the ability to yield chromosomal aberrations correlate with their cytotoxicity (Vogel *et al.*, 1996; 1998). Consequently, crosslinking agents are primarily clastogens rather than mutagens.

ICLs can be sensed by different mechanisms depending on the type of DNA transaction taking place on the crosslinked DNA molecule. In actively dividing cells, the replicative machinery will encounter an ICL during S-phase and the ICL will act as a physical barrier to replisome progression. This is thought to be a prevalent mechanism for sensing ICL lesions. In this setting, ICL repair involves the collision of a replication fork as a trigger to initiate repair. Upon sensing of an ICL during DNA replication, checkpoint activation, multistep repair and stabilization of the replisome must be tightly coordinated. ICL repair in vertebrates involves the generation of fragile DNA intermediates including DSBs, nicks and ssDNA gaps. If these DNA structures are left unrepaired or partially repaired, they may lead to replication fork collapse and aberrant recombination events such as chromosomal fusions that result in the radial chromosomes seen in FA patients.

It has been assumed that this replication-dependent mode of repair should be sufficient to cope with and repair ICL in a timely fashion. However, in non-proliferating cells such as post-mitotic differentiated cells or quiescent stem cells, endogenously generated ICLs need to be repaired in the absence of DNA replication. In this situation, if the ICL is positioned in an actively transcribed gene, a similar sensing process could take place following stalling of the RNA polymerase and transcription block could be the initiating event. However, much less is known about the consequences of blocked transcription by ICLs. Finally, it is thought that distorting ICLs (Table 2) can be recognized in the absence of

Table 2. Properties of different classes of crosslinking agents. Nucleotides in bold characters show the crosslinked nucleotides in ICLs.

	Nitrogen mustards	Platinum compounds	Nitrosoureas	Mitomycin C	Psoralens
Example of compounds	HN2, melphalan, chlorambucyl	Cisplatin, carboplatin	BCNU, carmustine	MMC	Psoralen, 8-methoxypsoralen
Crosslinked sequences (ICL)	5'-GNC-3' 3'-CNG-5'	5'-GC-3' 3'-CG-5'	5'-G-3' 3'-C-3'	5'-CG-3' 3'-GC-5'	5'-TA-3' 3'-AT-5'
Distortion	Variable	Major + bending	Unknown	Modest	Major, no bending
Fraction of ICLs (%)	1-5	2-5	3-8	5-13	Up to 40
Other major lesions	Mono-adducts	Intrastrand crosslinks	Mono-adducts, intrastrand crosslinks	Mono-adducts, ROS	Mono-adducts
Chemotherapeutic uses	Lymphoid tumors	Ovarian, testicular, lung	Glioblastoma	Gastrointestinal, breast, bladder	Vitiligo, psoriasis, cutaneous T cell lymphoma
References	Rink and Hopkins (1995); Dy and Adjei (2008)	Huang <i>et al.</i> (1995); Coste <i>et al.</i> (1999); Kartalou and Essigmann (2001)	Colvin <i>et al.</i> (1976); Wiencke and Wiemels (1995)	Rajski and Williams (1998); Warren <i>et al.</i> (1998); Pagano (2002)	Averbeck <i>et al.</i> (1988); Honig <i>et al.</i> (1994); Spielmann <i>et al.</i> (1995); Stern (2007)

collision with the replication or transcription machinery moving along DNA.

Crosslinking agents and their relevance

ICLs are generated by bifunctional agents that fall in different classes. Nitrogen mustards (Bis(2-chloroethyl) methylamine:HN2, chlorambucil, melphalan) have been used for over 65 years and are still widely used compounds for cancer therapy (Gilman and Philips, 1946), in particular for lymphoid tumors (multiple myeloma, chronic lymphocytic leukemia (CLL)). They react with the N7 of guanine to form primarily mono-adducts to DNA. However, 1–5% of the lesions are ICLs that are responsible for the cytotoxicity of these compounds (Rink and Hopkins, 1995).

Cisplatin (cisplatinum diammine chloride II, CDDP) and di- and trinuclear *cis*-DDP analogs were first identified as inhibitors of bacterial division (Rosenberg *et al.*, 1965) and are used to treat a wide spectrum of solid tumors with the greatest impact seen in the treatment of testicular and ovarian cancers. As with nitrogen mustards, ICLs represent a small fraction of lesions induced by platinum drugs (1–5%), which are primarily intrastrand crosslinks (Jones *et al.*, 1991; Brabec and Leng, 1993; Kartalou and Essigmann, 2001). These compounds also react with the N7 of guanine to form ICLs. While ICLs are thought to be the primary cause for cytotoxicity (Roberts and Friedlos, 1987), it is worth noting that intrastrand crosslinks are lesions that will trigger a checkpoint and activate DNA repair pathways overlapping with ICL repair. Notably, differences in cytotoxicity and efficacy between platinum compounds generating distinct intrastrand crosslinks have been reported. For example, trans-DDP, a less toxic analog of *cis*-DDP cannot form 5'-GG-3' intrastrand crosslinks (Aris and Farrell, 2009).

Mitomycin C (MMC) is an antibiotic from *Streptomyces* that is widely used in cell-based studies of ICLs (Tomasz, 1995). MMC needs to be metabolized to active intermediates, which trigger 5–14% of ICLs, among other adducts (Tomasz, 1995). MMC also generates reactive oxygen species (ROS), which contribute to its toxicity (Pagano, 2002). MMC is used to treat gastrointestinal, breast, lung and bladder cancers. Another class of antibiotic from *Streptomyces* are Pyrrolo[2,1-*c*][1,4]benzodiazepines (PBD) that form ICLs very efficiently (Gregson *et al.*, 2001; Clingen *et al.*, 2005).

Nitrosoureas (BCNU, carmustine) are chemotherapeutic agents that are metabolized to bifunctional molecules, which in turn generate less than 8% ICLs among other adducts (Wiencke and Wiemels, 1995). However, toxicity has limited the clinical use of nitrosoureas.

Finally, psoralen and its derivatives form interstrand crosslinks with DNA upon activation by UV irradiation with long-wavelength UV light. Psoralens are used for

the treatment of psoriasis, vitiligo, and cutaneous T-cell lymphoma. They form the highest fraction of ICLs (up to 40%) among crosslinking agents (Averbeck *et al.*, 1988; Dronkert and Kanaar, 2001; Honig *et al.*, 1994; Warren *et al.*, 1998).

The pathways of ICL repair have been primarily inferred through the use of these crosslinking agents. However, a variety of synthetic ICLs have also been used for *in vitro* studies and studies in cell-free extracts (see below). The development of systems utilizing DNA templates harboring single, defined ICL lesions offers significant insight into the mechanisms of ICL detection, signaling, and repair. This strategy is a significant advance over the use of crosslinking agents that generate only a minority of *interstrand* crosslinks and primarily generate *intrastrand* crosslinks and other forms of DNA damage that are capable of activating checkpoint signaling and activating repair mechanisms (Table 2).

It has been shown that ICLs can arise endogenously in cells (Niedernhofer *et al.*, 2003). Proposed sources of endogenous ICLs include aldehydes such as malondialdehyde (Kozekov *et al.*, 2003; Stone *et al.*, 2008). These compounds, which act essentially as bifunctional alkylating agents, are products of lipid peroxidation. Another source of ICLs is following the action of psoralen-related furocoumarins, which might be found in plants (Kleiner *et al.*, 2003). The extent to which endogenous ICLs form in cellular DNA and their possible biological effects remain unclear; however, it has been inferred that as many as 10 ICLs could be generated and repaired per cell each day (Lindahl and Barnes, 2000). Accumulation of ICL DNA lesions with time could contribute to the aging of cells (Grillari *et al.*, 2007).

The strongest evidence supporting the idea that ICLs are the toxic lesions among the DNA adducts generated by crosslinking agents comes from comparative studies between bifunctional compounds that are capable of forming ICLs and their chemically related monofunctional analogs, which are limited to the generation of monoadducts. Whereas monofunctional alkylating agents largely produce point mutations that are easily explained by the type of alkylation product formed, bifunctional crosslinking agents cause gross chromosomal alterations. For example, studies comparing chlorambucil with its monofunctional analog show that while treatment with chlorambucil, a nitrogen mustard that elicits ICL formation, resulted in deletions, its monofunctional analog caused point mutations (Yaghi *et al.*, 1998). Furthermore, ICL-generating agents are more effective at inducing chromosomal losses and sister chromatid exchanges than their monofunctional analogs (Bodell *et al.*, 1985; Vogel *et al.*, 1996).

Table 2 summarizes characteristics of some of the crosslinking agents used in cancer therapy and/or for experimental studies of ICLs. Notably, different

crosslinking sources yield different ranges of aberrant DNA structures of which ICLs always compose the less abundant fraction. Moreover, ICLs have unique structural characteristics. In particular, the physico-chemical nature of an ICL influences the topology of the surrounding DNA helix, which in turn is thought to affect the manner in which the ICL is recognized and repaired (Rajski and Williams, 1998; Noll *et al.*, 2006; Smeaton *et al.*, 2008; 2009).

Model systems to study ICL repair

Some of the characteristics of the different strategies for repairing ICLs are outlined below. Please see Dronkert and Kanaar (2001), Niedernhofer *et al.* (2005), Patel and Joenje (2007) and McVey (2010) for more complete reviews. ICL repair was first investigated in *E. coli* (Cole, 1973). There, ICL repair initiates by incisions on either side of the ICL by Uvr(A)2BC (Van Houten *et al.*, 1988; Sladek *et al.*, 1989). If homologous recombination is available, resection at one of the nicked positions elicits RecA loading and invasion of the undamaged template followed by DNA synthesis across the region containing the crosslink. Uvr(A)2BC then excises the unhooked crosslink and the resulting gap is filled. When HR is not available, error-prone translesion synthesis (via Pol II) occurs following Uvr(A)2BC incisions. ICL repair in yeast uses similar strategies but, in addition, is tightly cell cycle regulated (McHugh *et al.*, 2001). Of note, models of ICL repair in *S. cerevisiae* are primarily based on genetic data and the sequence of events remains hypothetical. During S-phase, the collision of a replication fork with an ICL results in the generation of a DSB (McHugh *et al.*, 2000). This DSB could arise as a consequence of nucleolytic cleavage following fork stalling by the ICL or could result from the fork encountering a single-stranded nick generated by a pre-existing incision in the proximity of the ICL. The ICL is then unhooked and, following translesion synthesis, removed by the nucleotide excision repair (NER) pathway. Additionally, the HR pathway is required for repair of the DSB intermediate, as strongly suggested by the sensitivity of HR mutants to crosslinking agents (Henriques and Moustacchi, 1981). In contrast, cells in G1 lack moving replication forks to sense ICLs and lack replicated homologous sequences to use in a HR step in repair. In G1, ICL recognition is dependent on the NER machinery (Lehoczky *et al.*, 2007) and is followed by translesion synthesis and a second set of incisions releasing the crosslinked nucleotide. The occurrence of ICL repair in G1 in yeast is supported by the mutagenic nature of repair and by the sensitivity of yeast harboring mutations in error-prone post-replication repair to crosslinking agents (Henriques and Moustacchi, 1981; McHugh *et al.*, 2000). Notably, yeast use different sets of repair pathways to repair lesions induced by different

crosslinking agents (Beljanski *et al.*, 2004). This suggests that the structure of the crosslink influences, at least in part, the mode of repair.

ICL repair in higher eukaryotes is less understood. However, some fundamental similarities are thought to exist between yeast and higher eukaryotes. ICL repair is accompanied by DSB formation that requires HR for repair. While HR- and replication-dependent ICL repair is thought to be the prominent mode of repair, replication-independent, HR-independent, and error-prone pathways of ICL repair have also been described (Wang *et al.*, 2001; Zheng *et al.*, 2003). In particular, it has been shown that psoralen crosslinks induced by UV laser irradiation are repaired in G1 cells by a XPC-dependent repair mechanism (Muniandy *et al.*, 2009). Furthermore, it has been shown, using quantitative RT-PCR across repaired lesions, that a plasmid harboring a single ICL is repaired in G1-arrested mammalian cells (Ben-Yehoyada *et al.*, 2009, and see below).

A fundamental difference between yeast and other organisms is the requirement for FA proteins. The helicases FANCD1 and FANCD2 appeared first in evolution and have homologs in bacteria and Archaea (Meetei *et al.*, 2005; White, 2009). Invertebrates have several FA genes with a FANCD2/I complex and downstream FA genes: FANCD1 and FANCD2 in *Caenorhabditis elegans* and FANCD1 in *Drosophila*. While *Drosophila* has a FANCL homolog, *C. elegans* appears to be devoid of FA core complex proteins raising the question of the identity of the ubiquitin ligase responsible for FANCD2 ubiquitylation, which is observed in *C. elegans* (McVey, 2010).

Most studies on FA and ICL repair have been performed in human and mouse cells as well as in mouse models and in chicken lymphoblastic cells (DT40). In addition, cell-free extracts from *Xenopus* eggs have recently emerged as a powerful system to study ICL repair and signaling from ICLs.

FA proteins in the ICL damage response

FA proteins as sensors for DNA damage

The FA pathway has been implicated in the response to DNA damage, including but not limited to ICL damage. Several FA proteins are modified following DNA damage, including the monoubiquitylation of FANCD2 and FANCI (Figure 1). In addition to their role downstream of checkpoint kinases, it has been speculated that FA proteins could also play a role in the early steps of the DNA damage response, i.e. as sensors for DNA damage.

The FA pathway has been shown to be an early responder to ICL damage. FA cells display a defect in the initial ICL incision step, generally attributed to XPF/ERCC1 activity (Fujiwara, 1982; Kumaresan and Lambert,

2000; Kumaresan *et al.*, 2007), although XPF could have additional functions (reviewed in Bergstralh and Sekelsky, 2008; Hlavin *et al.*, 2010). This has implicated the FA pathway in the sensing of ICLs and/or the recruitment of XPF/ERCC1. This idea was recently supported by experiments in *Xenopus* cell-free extracts showing that FANCD2 depletion resulted in an incision defect and subsequent delay in translesion synthesis (Knipscheer *et al.*, 2009).

Experiments using small plasmid templates harboring a single ICL have considerably strengthened the idea that FA proteins could play a direct role in sensing ICLs. Shen *et al.* (2009) employed a substrate harboring an EBV replication start site and a psoralen-induced ICL and found that FA proteins are enriched at the ICL site. Importantly, in these experimental conditions, FA core complex components and FANCD2/FANCI are recruited to the ICL in a replication-independent manner, implicating the FA pathway in an early step in the sensing of an ICL. In contrast, the recruitment of the downstream FA proteins FANCD1, FANCF and FANCG requires replication but not the FA core complex, suggesting that the FA core and FANCD2/FANCI proteins are recruited directly to ICL and the BRCA-related FA proteins are recruited to the stalled replication fork at the ICL (Shen *et al.*, 2009). The differential recruitment of FA proteins could reflect the involvement of the FA pathway in distinct ICL repair pathways and is consistent with a sensing role for FA proteins in replication-independent ICL repair and a role of the “downstream” FA proteins in HDR, which takes place in replication-dependent repair of ICLs. Consistent with this, using cell-free extracts from *Xenopus*, it was shown that FA proteins are recruited to a plasmid harboring a single distorting ICL regardless of whether or not DNA replication is taking place (Ben-Yehoyada *et al.*, 2009). It was also demonstrated that the recruitment of FA proteins was independent of the RPA- and ATR-dependent checkpoint (Ben-Yehoyada *et al.*, 2009). These data implicate the FA pathway in an early step in the sensing of an ICL, upstream of checkpoint signaling. This may explain why FA cells are so exquisitely sensitive to ICLs since they are unable to properly recognize ICLs and therefore unable to activate the subsequent necessary steps to remove the ICL.

FA proteins and ICL repair

The hypersensitivity to crosslinking agents shared by all FA patients indicates that the FA pathway plays an essential role in sensing, signaling from, or repairing lesions generated by these agents. However, the exact role of the FA proteins in the response to ICLs still remains largely elusive. The FA pathway could participate in both replication-dependent and -independent pathways of ICL repair outlined above. When FA cells are exposed to crosslinking

agents, they accumulate chromosomal breaks and radial chromosomes (Auerbach, 1993), an outcome that indicates a defect in the cellular response to ICLs.

After recognizing the ICL and signaling cell cycle arrest to allow its repair, the FA pathway may then function to coordinate the repair of the ICL. Disruption of the FA core complex and the ID complex has been shown to decrease ICL repair efficiency (Ben-Yehoyada *et al.*, 2009; Shen *et al.*, 2009). The mechanisms of ICL repair are believed to involve HDR, translesion synthesis (TLS), and part of the NER machinery. Whereas TLS and nuclease(s) are required for both replication-dependent and -independent repair, homology-dependent repair is thought to be required only in replication-dependent repair when sequences homologous to the lesion are available. However, it is also conceivable that replication-independent, HDR-dependent ICL repair could operate in G2 phase of the cell cycle. Notably, the FA pathway has been associated with proteins involved in HDR, TLS and NER. The exact role of FA proteins in HDR is unclear. While FANCD1 cells show a marked defect in HDR (Moynahan *et al.*, 2001; Stark *et al.*, 2004; Nakanishi *et al.*, 2005), other FA cells show a milder HDR defect (Yamamoto *et al.*, 2003; 2005; Niedzwiedz *et al.*, 2004; Hirano *et al.*, 2005; Nakanishi *et al.*, 2005; Yang *et al.*, 2005). However, the link between the FA pathway and the BRCA pathway is well established since FANCD1 is identical to BRCA2 and several FA pathway proteins interact directly with BRCA1 or BRCA2. Therefore, it is possible that while the FA pathway does not play a major role in all HDR, it plays a specific role in the recruitment of repair proteins and the coordination of HDR repair in the context of ICL damage. Indeed, *in vitro* studies suggest that FANCD1/BRCA2 play a significant role in ICL repair (Cipak *et al.*, 2006). FANCD2 also associates with the Mre11-Rad50-Nbs1 (MRN) complex, which is essential for DNA resection at DSBs, the first step of all homology-dependent repair processes (Roques *et al.*, 2009). MRN is required for FANCD2 foci formation at sites of damage: ssDNA gaps and DSBs (Roques *et al.*, 2009).

In response to crosslinking agents, FANCD2 has been shown to colocalize with NER component XPF (Sridharan *et al.*, 2003) and XPF affects the stability of ubiquitylated FANCD2 (Bhagwat *et al.*, 2009). FANCD2 has also been shown to colocalize with TLS polymerase Rev1 after replication arrest (Niedzwiedz *et al.*, 2004) and FA core complex components FANCA and FANCG have been shown to be required for efficient Rev1 foci formation (Mirchandani *et al.*, 2008). Playing an ICL-specific role in HDR and a role upstream of TLS and NER, the FA pathway helps to coordinate and regulate these repair mechanisms for the efficient and proper removal of ICL damage. Consistent with these ideas, it was shown that inactivation of FANCD2 affected both nucleolytic incision and translesion synthesis (Knipscheer *et al.*, 2009).

Recent experiments have examined the role of the FA pathway in ICL repair using DNA substrates containing a single, site-specific ICL in cell-free extracts from *Xenopus* (Raschle *et al.*, 2008; Ben-Yehoyada *et al.*, 2009). In addition, the behavior of ICL-containing DNA templates was monitored in mammalian cells upon transfection (Ben-Yehoyada *et al.*, 2009; Shen *et al.*, 2009).

One of the *Xenopus* studies reported a system in which ICL repair is exclusively replication-dependent (Raschle *et al.*, 2008; Knipscheer *et al.*, 2009). This study describes in detail the initial steps in ICL processing in a replication-dependent system using primarily a nitrogen mustard-like ICL adduct that minimally distorts the DNA helix (Raschle *et al.*, 2008). Two replication forks converge on the ICL with the leading strand stalling initially 20–24 nucleotides from the ICL, followed by the advancement of one leading strand to within 1 nucleotide of the crosslink. Notably, these findings suggest that translesion synthesis precedes ICL unhooking. This model also departs from earlier models invoking the collision of a single replication fork with an ICL (Niedernhofer *et al.*, 2005). On a small plasmid DNA template, convergence of two forks on the ICL is the rule. However, on a chromosome, it is not clear how often this phenomenon will take place. The distance between origins of replication ranges from 150 to 500 kb in somatic cells and given that ICLs activate a checkpoint that will decrease the density of active origins, it is reasonable to think that a significant fraction of ICLs will be met by a single replication fork. Therefore these two modes of replication-dependent ICL repair might co-exist.

Using a DNA substrate containing a MMC-like ICL adduct that significantly distorts the DNA helix, another study found that ICL repair can proceed through replication-dependent and -independent mechanisms (Ben-Yehoyada *et al.*, 2009). Repair is associated with a significant amount of DNA synthesis (Ben-Yehoyada *et al.*, 2009). The observation that ICL repair could take place in the absence of DNA replication in *Xenopus* extracts and upon transfection of an ICL-containing plasmid in G1-arrested mammalian cells (Ben-Yehoyada *et al.*, 2009) is consistent with accumulating evidence for ICL repair in G1, discussed above (Sarkar *et al.*, 2006; Hlavin *et al.*, 2010; Muniandy *et al.*, 2010; Shen and Li, 2010). Some of the differences reported in *Xenopus* could stem from the different types of ICLs used. As mentioned above, yeasts use different repair pathways to repair ICLs generated by different crosslinking agents (Beljanski *et al.*, 2004). Similarly, it has been shown that in mammalian cell extracts, the level of unhooking of a 5'-GC-3' ICL (distorting) was 10-fold greater than for a 5'-CG-3' ICL (non-distorting). These results demonstrate that helix distortion influences significantly ICL recognition and subsequent repair (Smeaton *et al.*, 2008).

The development of site-specific ICL template systems offers great promise for the future of ICL research as these substrates provide the opportunity to examine ICL processes specifically in the absence of other forms of DNA damage. The use of these substrates will help delineate the molecular steps involved in ICL processing and the proteins recruited. The site-specific nature of these ICL substrates allows for the determination of repair efficiency and analysis of repair intermediates and repair errors. In addition, the ability to introduce site-specific ICL adducts into mammalian cells allows for *in vivo* studies of ICL repair and signaling. This should also provide a prognostic tool to assess the ability of cancer patients' cells to repair ICLs and subsequently to predict the patients' response to crosslinking drugs. Likewise, this tool may be used to assess the efficacy of therapeutics targeting crosslink repair.

FA proteins and checkpoint activation

The FA pathway has been connected to checkpoint activity in multiple ways. The complexity of these interactions is seen by the fact that FA is involved upstream and downstream of checkpoint kinase signaling. Studies in *Xenopus* cell-free extracts using a DNA substrate containing a 5'-GC-3' ICL adduct that distorts the DNA helix, found that the ICL activates robust replication-dependent and -independent checkpoints. Following recognizing ICL damage, the FA pathway plays an essential role in the induction of an ATR/Chk1-dependent checkpoint (Ben-Yehoyada *et al.*, 2009). This is consistent with the replication-independent specific association of FA core and ID complexes to a psoralen ICL (Shen *et al.*, 2009). Depletion of a FA core protein (FANCL) or of FANCD2 impairs checkpoint signaling. Checkpoint activity was measured by the ability of ICL-containing DNA to inhibit the replication of an undamaged plasmid *in trans*. Inhibition of the FA pathway also inhibited phosphorylation of CHK1. Consistent with this finding, depletion of FA proteins prevented the recruitment of RPA and ATR to the ICL. This establishes that the FA pathway operates upstream of the RPA-ATR-CHK1 signaling pathway. Importantly, recruitment of RPA and ATR to plasmids harboring UV lesions is not affected by FA pathway inhibition, thus identifying an ICL-specific signaling function for the FA pathway (Ben-Yehoyada *et al.*, 2009). The generation of ssDNA-RPA intermediates upon encountering an ICL with a progressing replication fork can be envisioned as a consequence of fork stalling and processing of the ICL (Figure 2). In contrast, the mechanism responsible for generating ssDNA-RPA in the absence of DNA replication is unknown (Figure 2). In particular, it remains to be determined whether the recruitment of FA proteins is a prerequisite for unhooking of the ICL and/or

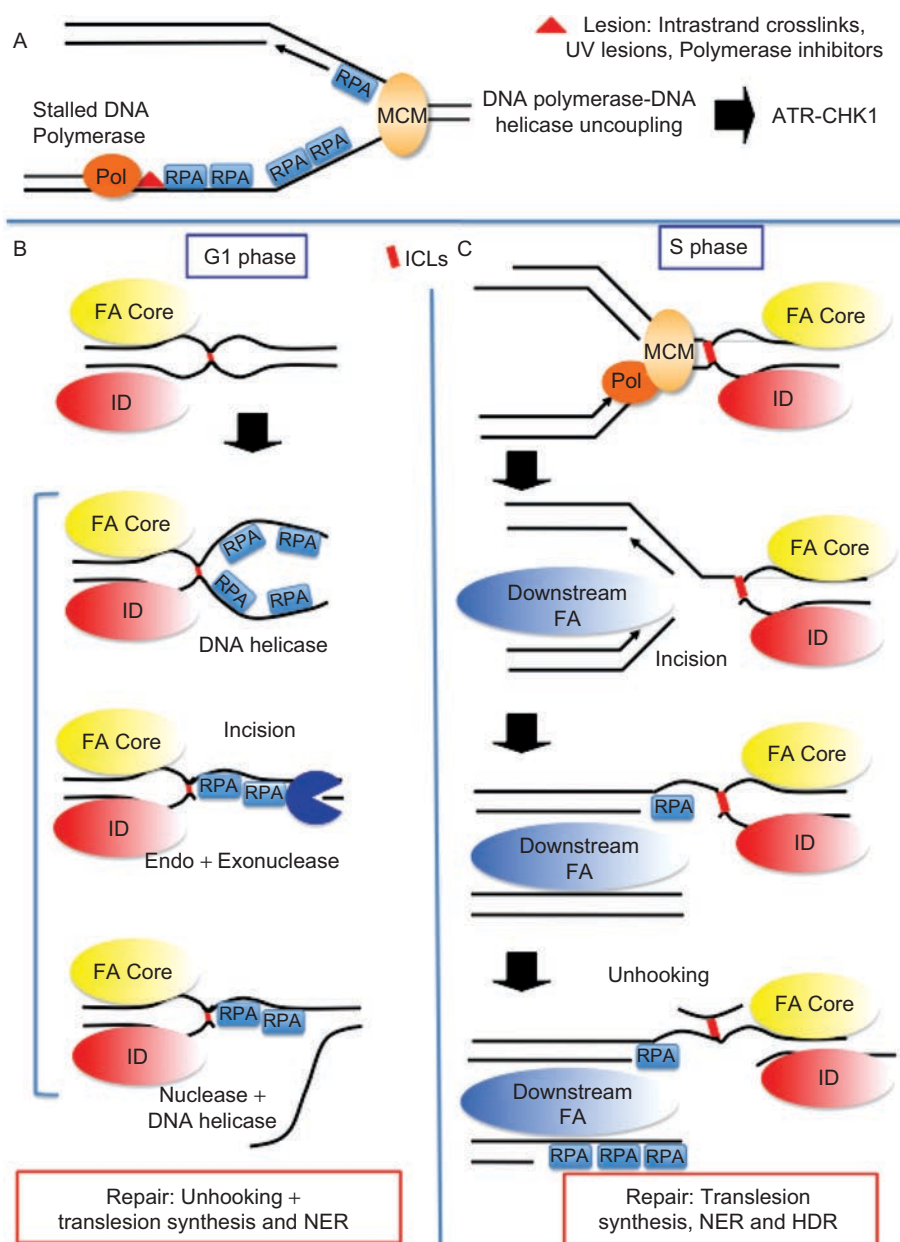


Figure 2. Cell cycle regulation of signaling from crosslink and crosslink repair. (A) Canonical replication checkpoint activated by a lesion on one strand of the DNA. The lesion results in DNA polymerase stalling that becomes uncoupled from the MCM DNA helicase. The resulting ssDNA-RPA intermediates activate the ATR-CHK1 checkpoint pathway (Byun *et al.*, 2005). This checkpoint is activated in response to UV lesions, DNA polymerase inhibition but also intra-strand crosslink generated by crosslinkers such as cisplatin. (B) Checkpoint activation and ICL repair in G1. In G1, the core and ID complexes associated with ICL damage and a checkpoint is activated. It is not known whether ssDNA-RPA accumulates prior to, or as a consequence of, an incision in the proximity of the crosslink. Several hypothetical modes of ssDNA-RPA are presented. For clarity, accumulation of ssDNA-RPA is shown only on one side of the ICL, but could take place on both sides. Repair will proceed following unhooking, via translesion synthesis and NER. (C) Checkpoint activation and ICL repair in S-phase. In S-phase, FANCD1/FANCI/FANCN (downstream FA) associate with the fork stalled at the ICL independently of the core and ID complexes. Hypothetically, ssDNA-RPA could be generated following the incision and/or unhooking of the ICL on the lagging strand. In addition, the resected DSB generated following incision could be an additional source of ssDNA-RPA to activate a checkpoint. Note that the MCM helicase is thought to be loaded around dsDNA and therefore unable to unwind DNA passed the ICL. Therefore, the mechanism of checkpoint activation at an ICL is presumably distinct from the polymerase-helicase uncoupling described in (A). Repair will proceed following unhooking, via translesion synthesis, NER and HDR.

whether incision(s) at the ICL is required for checkpoint activation.

FANCM is involved in the ATR-mediated checkpoint response, independently of the FA core complex (Collis *et al.*, 2008). In the absence of FANCM, TOPBP1 is not retained on chromatin and phosphorylation of ATR downstream targets is defective (Luke-Glaser *et al.*, 2010; Schwab *et al.*, 2010). Interestingly, FANCM appears to be central to a feedback loop in checkpoint activation as CHK1 prevents FANCM proteasome-dependent degradation upon DNA damage.

FA proteins are also required downstream of checkpoint kinases for the proper execution of checkpoint signaling. This was first demonstrated by showing that phosphorylation of FANCD2 by ATM following IR was required for the activation of an S-phase checkpoint (Taniguchi *et al.*, 2002b). Similarly, it was shown that the FA core complex and FANCD2 regulate the ATR-dependent intra-S-phase checkpoint in response to distorting ICLs induced by psoralen (Pichierri and Rosselli, 2004a; 2004b). In contrast, other studies suggested that the FA pathway does not regulate the intra-S-phase checkpoint following treatment with MMC that generate non-distorting ICLs (Andreassen *et al.*, 2004). These differences could reflect distinct responses from cells deficient in different FA genes and/or different responses to different crosslinking agents yielding ICL lesions with different properties (Table 2).

Checkpoint pathways also regulate the FA pathway and, in particular, FANCD2 activation. ATR is required for the efficient ubiquitylation of FANCD2 (Andreassen *et al.*, 2004). Both ATR and BRCA1 were shown to facilitate FANCD2 ubiquitylation in response to rereplication (Zhu and Dutta, 2006b). The FA pathway appears to participate in a feedback loop in response to rereplication since the FA core complex, but not FANCD2, is required for checkpoint activation in rereplicating cells (Zhu and Dutta, 2006a).

The FA pathway and DNA replication

The FA pathway is not only activated following treatment with crosslinking agents, but is also responsive to treatments that affect DNA replication (such as the induction of replication stress). The FA pathway is also activated during normal S-phase and the ID complex binds to chromatin during S-phase, in the absence of exogenous DNA damage (Mi and Kupfer, 2005; Sobeck *et al.*, 2006). Disruption of the FA pathway leads to the accumulation of DSBs in the absence of exogenous damage (Sobeck *et al.*, 2006), suggesting that the FA pathway plays a role in preventing the accumulation of damage during unperturbed DNA replication. The nature of the DNA structure(s) that are recognized and processed by the FA pathway during DNA replication remains elusive.

In cell-free extracts, the FA pathway, as seen by FANCD2 ubiquitylation, is activated by various structures (Sobeck *et al.*, 2007).

The FA core complex interacts with BLM, topoisomerase II α , and RPA in the BRAF complex (Meetei *et al.*, 2003b) and is required for ICL-dependent BLM phosphorylation and recruitment to nuclear foci (Pichierri *et al.*, 2004). Both ATR and BLM have been shown to function at stalled replication forks to stabilize the forks during repair processes and prevent the collapse of the replication fork. Disruption of the FA pathway increases the expression of fragile sites as well as the number of breaks and gaps at known fragile sites (Howlett *et al.*, 2005). FANCD2, FANCI and BLM proteins associate with ultrafine bridges (UFB) during mitosis. These structures are thought to reflect improper replication of fragile site loci (Chan *et al.*, 2009). Moreover, FANCI and FANCM are enzymes that can process DNA structures arising at replication forks (Wu *et al.*, 2008; Sommers *et al.*, 2009; Schwab *et al.*, 2010). ATM and ATR can phosphorylate a number of FA proteins and many of these phosphorylations are required for efficient activation of the FA pathway during DNA replication (Ho *et al.*, 2006; Matsuoka *et al.*, 2007; Qiao *et al.*, 2004; Wang *et al.*, 2007; Yamashita *et al.*, 1998).

FA proteins can bind specifically at the DNA replication fork where they are poised to detect and respond to DNA damage and stabilize the replication fork (Wang *et al.*, 2008). Disruption of the FA pathway following inactivation of FANCL leads to a defect in replication restart at collapsed replication forks after treatment with MMC or camptothecin (CPT), a topoisomerase I inhibitor (Wang *et al.*, 2008). Similarly, cells deficient for FANCM are defective in DNA replication restart following CPT treatment (Luke-Glaser *et al.*, 2010; Schwab *et al.*, 2010). While MMC generates ICLs (among other DNA lesions) and CPT ultimately leads to the generation of DSBs, both treatments lead to the formation of DNA lesions blocking the replication fork. The requirement for the FA pathway in replication restart after both treatments indicates that the FA pathway functions to stabilize the replication fork when it encounters a physical lesion blocking the fork and/or to coordinate the reassembly of the replication fork and the restart of replication after the removal of the lesion.

FA proteins in cancer

The FA pathway is connected to cancer in many ways. First, Fanconi anemia is associated with cancer predisposition. Second, because the FA pathway is critical for ICL sensing and repair, its integrity determines the sensitivity or resistance to chemotherapeutic drugs that generate ICLs.

The relationship between Fanconi anemia and cancer predisposition has been well established. Almost 25%

of FA patients develop malignancies, especially myelodysplastic syndrome, acute myelocytic leukemia, and squamous cell carcinomas of the head and neck (Kutler *et al.*, 2003). However, more recently, FA proteins have been implicated in familial and sporadic cancers outside the FA patient population. FANCD1, FANCF and FANCD2 have been implicated in breast cancer susceptibility as truncating mutations in each of these genes have been identified (Seal *et al.*, 2006; Erkkö *et al.*, 2007; Rahman *et al.*, 2007; Reid *et al.*, 2007; Xia *et al.*, 2007; Turnbull and Rahman, 2008). FANCD1 mutations have also been associated with ovarian, prostate, stomach and pancreatic cancers (Friedenson, 2005) and FANCD2/PALB2 truncations have been implicated in prostate cancer (Erkkö *et al.*, 2007). RAD51C, which was recently implicated in a FA-like disorder (Vaz *et al.*, 2010), is also involved in breast and ovarian cancer susceptibility (Meindl *et al.*, 2010). Monoallelic, dominant mutations were observed in families with both breast and ovarian cancer, reminiscent of families with BRCA2 deficiencies (Levy-Lahad, 2010; Meindl *et al.*, 2010). Promoter methylation has also been identified as a method of FA inactivation. FANCF promoter methylation was first found to be associated with ovarian cancer by Taniguchi *et al.* (2003) and this finding has been confirmed by several other groups (Dhillon *et al.*, 2004; Wang *et al.*, 2006). In addition to ovarian cancer, FANCF promoter methylation has been implicated in acute myeloid leukemia (Tischkowitz *et al.*, 2003), multiple myeloma (Hazlehurst *et al.*, 2003; Chen *et al.*, 2005), bladder cancer (Neveling *et al.*, 2007), cervical cancer (Narayan *et al.*, 2004), head and neck carcinomas and non-small cell lung cancers (Marsit *et al.*, 2004). Promoter methylation of other FA core complex members, FANCC, FANCG, and FANCL, have also been implicated in pancreatic cancer (van der Heijden *et al.*, 2003; Rogers *et al.*, 2004; Couch *et al.*, 2005), breast cancer (Sinha *et al.*, 2008) and leukemia (Hess *et al.*, 2008). Promoter methylation of PALB2 has been reported in breast tumors (Potapova *et al.*, 2008). In addition, altered expression of FANCL due to a splice variant was found in Calu-6 lung cancer cells (Zhang *et al.*, 2006), and a FANCD2 SNP was found to be associated with sporadic breast cancer in the Spanish population (Barroso *et al.*, 2006). Genetic inactivation and epigenetic silencing of the FA pathway in sporadic cancers underscore the critical tumor suppressor function of this pathway.

The response of cancer cells, either sensitivity or resistance, to DNA damaging drugs such as the crosslinking agent cisplatin can vary depending on the status of certain DNA repair pathways. For example, cisplatin sensitivity in testicular germ cells and non-small cell lung cancer cells is associated with decreased expression of nucleotide excision repair proteins XPA and ERCC1, respectively (Koberle *et al.*, 1999; Welsh *et al.*, 2004; Fujii *et al.*, 2008; Vilmar and Sorensen, 2008). In addition,

secondary mutations in BRCA1 mediate cisplatin resistance in BRCA1-mutated ovarian tumors (Swisher *et al.*, 2008). Therefore, the status and modulation of DNA repair pathways can play a role in tailoring chemotherapy regimes and re-sensitizing cisplatin-resistant cancers to treatment, respectively.

Like cells from FA patients, many of the cancer cells lines and primary tumor samples with a disruption in the FA pathway show crosslinker sensitivity. Inactivation of the FA pathway has been associated with increased sensitivity to crosslinking agents such as cisplatin, MMC, and melphalan in ovarian (Taniguchi *et al.*, 2003), pancreatic (van der Heijden *et al.*, 2004; 2005; Sakai *et al.*, 2008), bladder (Neveling *et al.*, 2007), multiple myeloma (Yarde *et al.*, 2009) and lung cancers (Zhang *et al.*, 2006). In addition, defects in the FA pathway have been associated with sensitivity to alkylating agents in glioma cells (Chen *et al.*, 2007). The FA pathway defect was found to be directly responsible for chemosensitivity since restoration of an intact FA pathway abolished the sensitivity and led to chemoresistance (Taniguchi *et al.*, 2003; van der Heijden *et al.*, 2005). Several studies have also shown that the targeted disruption of the FA pathway by deletion of FANCC or FANCG, the introduction of dominant-negative FANCA, siRNA targeting of FANCF or FANCD2, or inhibition by the small molecule inhibitor curcumin directly leads to chemosensitization to cisplatin, melphalan, alkylating agents, and PARP inhibitors (Ferrer *et al.*, 2004; Chen *et al.*, 2005; 2007; Gallmeier *et al.*, 2006; Sakai *et al.*, 2008). Finally, inactivation of BRCA2/FANCD1 has been associated with sensitivity to cisplatin in ovarian carcinoma. The connection between FA pathway disruption and chemosensitivity identifies the modulation of the FA pathway as a means of chemosensitization. Likewise, identifying FA pathway defects in tumors can lead to the generation of targeted cancer therapy regimes that take advantage of this tumor-specific defect.

While FA pathway disruption leads to cisplatin sensitivity, reactivation of the pathway functions in the development of cisplatin resistance. Strikingly, *de novo* resistance of BRCA2-deficient ovarian tumor caused by reversion of BRCA2 point mutations has been described in patients and in cells in culture (Sakai *et al.*, 2008; 2009). *In vitro* incubation of cisplatin-sensitive cells containing a FANCD1/BRCA2 mutation or FANCF promoter hypermethylation also resulted in acquired cisplatin-resistance mediated by the restoration of the FA pathway (Taniguchi *et al.*, 2003; Sakai *et al.*, 2008). In the case of FANCF promoter methylation, treatment with demethylating agents restores cisplatin resistance (Taniguchi *et al.*, 2003). The processes of FA inactivation and reactivation shed light on the mechanisms of cisplatin sensitivity and resistance and also suggest new modalities of cancer therapy through inhibition of the FA pathway. A cell may initially take advantage of an inactivated FA pathway to generate

chromosomal instability and accelerate mutagenesis, during which time it is sensitive to cisplatin treatment. However, cisplatin treatment may select for revertant cells that have reactivated the FA pathway and are now cisplatin-resistant. Therefore, targeting the FA pathway may serve to re-sensitize these cells to cisplatin and can be used in combination with cisplatin to improve the efficacy of this treatment.

Screens have identified genes that, when deregulated, sensitize FA cells. Of note, both major branches of the DNA damage checkpoint pathways, i.e. ATM and ATR-Chk1, sensitize FA cells when downregulated (Kennedy *et al.*, 2007; Chen *et al.*, 2009). Similarly, a small molecule was described that renders FA cells hypersensitive to crosslinking agents (Gallmeier *et al.*, 2007). Several groups have also started the search for FA pathway inhibitors. Chirnomas *et al.* (2006) reported an initial finding of 4 FA/BRCA pathway inhibitors – wortmannin, H-9, alsterpaullone, and curcumin – from a high-throughput screen using human cells. Landais *et al.* (2008; 2009) developed a *Xenopus* cell-free extract based assay for FA inhibitors. Jacquemont and Taniguchi (2007) showed that proteasomal inhibitors also inhibit the FA pathway, and Burkitt and Ljungman (2008) showed that phenylbutyrate inhibits the FA pathway. The specific inhibition of the FA pathway offers a promising future for cancer therapies, allowing us to improve the efficacies of our current regimens and overcome the development of resistance.

Conclusions

The FA pathway plays a central role in ICL repair and the maintenance of genomic integrity. Functioning upstream of checkpoint signaling and repair mechanisms, the FA pathway acts as a sentinel in the detection of ICLs. Once an ICL is encountered, the FA pathway is activated and signals downstream to activate an ATR/Chk1-dependent checkpoint. The FA pathway also functions to coordinate HDR, TLS, and NER repair proteins in the repair of ICLs. During ICL recognition and repair, the FA pathway stabilizes the replication fork so that replication can recommence once the damage is repaired. In the absence of an intact FA pathway, cells are sensitive to spontaneous and DNA damage-induced chromosomal breaks.

Disruption of the FA pathway has been found in a number of cancers and may play a role in carcinogenesis by contributing to genomic instability. Inactivation of the FA pathway by promoter methylation and truncating mutations of FA proteins disrupts genomic integrity and contributes to cisplatin sensitivity in many cancers. However, cisplatin resistance may develop after initial treatments with cisplatin as the FA pathway becomes reactivated. The essential role of the FA pathway in

cisplatin sensitivity and resistance identifies the FA pathway as a specific target for cancer therapy. Assessing the status of FA pathway function can identify the specific tumors susceptible to crosslinking agents. In addition, targeted disruption of the FA pathway can restore cisplatin sensitivity to resistant tumors, and can also be used as combination therapy with a variety of chemotherapeutic agents including crosslinking agents, ionizing radiation, alkylating agents, and PARP inhibitors to increase the efficacy of these treatments.

The development of tailored chemotherapies and combination therapy with specific chemosensitizers that target DNA repair pathways hold great promise for the future of cancer treatment. However, it will be important to target these chemosensitizers to tumor cells to avoid the disruption of key DNA repair pathways in adjacent normal cells. In addition, the inhibitors should target key downstream proteins so as to avoid the development of compensating mechanisms leading to chemoresistance. The initial findings of small molecule inhibitors of the FA pathway and their implication in restoring cisplatin sensitivity identify the FA pathway as a DNA repair pathway that can be specifically targeted in this manner. As greater understanding of the roles and mechanisms of the FA pathway is achieved and specific inhibitors of this pathway are identified, targeted modulation of the FA pathway may play an important role in many chemotherapeutic regimes.

Declaration of interest

The authors acknowledge funding from the National Institute of Health (GM 077495 and CA92245 to J. G.)

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Editor: Michael M. Cox