REVIEW

The Fanconi Anemia/BRCA pathway: new faces in the crowd

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Over the past few years, study of the rare inherited chromosome instability disorder, Fanconi Anemia (FA), has uncovered a novel DNA damage response pathway. Through the cooperation of multiple proteins, this pathway regulates a complicated cellular response to DNA cross-linking agents and other genotoxic stresses. In this article we review recent data identifying new components of the FA pathway that implicate it in several aspects of the DNA damage response, including the direct processing of DNA, translesion synthesis, homologous recombination, and cell cycle regulation. We also discuss new findings that explain how the FA pathway is regulated through the processes of ubiquitination and deubiquitination. We then consider the clinical implications of our current understanding of the FA pathway, particularly in the development and treatment of malignancy in heterozygous carriers of FA mutations or in patients with sporadic cancers. We consider how recent studies of p53-mediated apoptosis and loss of p53 function in models of FA may help explain the clinical features of the disease and finally present a hypothesis to account for the specificity of the FA pathway in the response to DNA cross-links.

Fanconi Anemia (FA) is a rare autosomal recessive and X-linked disorder characterized by congenital abnormalities, progressive bone marrow failure, and cancer susceptibility (Joenje and Patel 2001; Bagby Jr. 2003; D'Andrea and Grompe 2003). Cells derived from FA patients demonstrate chromosomal instability and heightened sensitivity to DNA cross-linking agents, such as mitomycin C (MMC) (German et al. 1987), diepoxybutane (DEB) (Auerbach 1988), and cisplatin (CDDP) (Poll et al. 1985), a feature that is used to make the diagnosis. Most patients will develop bone marrow failure and can develop acute leukemia. Those who survive to adulthood typically develop solid tumors that are usually squamous cell carcinomas of the head and neck or gynecologic system (Alter 1996; Alter et al. 2003).

[**Keywords:** BRCA; cancer; chemotherapy; DNA cross-links; DNA helixase; Fanconi Anemia]

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1370505>.

FA is a genetically heterogeneous disease and has been traditionally classified using cell fusion experiments that correct sensitivity to DNA cross-linking agents such as MMC. To date at least 12 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M) have been identified (Table 1). Complementation groups A, C, and G are the most prevalent accounting for approximately 60%, 15%, and 10% of patients, respectively (Strathdee et al. 1992a; Lo Ten Foe et al. 1996; de Winter et al. 1998). The genes for 11 of the complementation groups have been cloned, and 10 of the proteins have been demonstrated to cooperate in a common biochemical pathway. Disruption of the FA pathway results in the common clinical and cellular phenotype observed in FA.

The basic FA pathway

During the S phase of the cell cycle, DNA is duplicated by DNA polymerases at multiple replication forks. DNA lesions that disrupt chromatin structure, such as chemically induced cross-links, result in a failure of the replication fork to proceed. As will be discussed later in this article, current evidence suggests that the FA pathway is activated and is required for the response to stalled replication forks. For the purposes of description it is easiest to consider the FA pathway as containing two main complexes, the nuclear E3 monoubiquitin ligase core complex (referred to as complex 1 in this review) and the chromatin-associated FANCD2/BRCA2 DNA repair complex (referred to as complex 2). As part of the response to a stalled replication fork, complex 1 may be largely involved in the detection and signaling of the stalled replication fork through monoubiquitination of FANCD2 whereas complex 2 may be predominantly involved in the process of DNA repair. It is important to note that the distinction between complex 1 and complex 2 is arbitrary and reflects a recognizable transition in the FA pathway (the monoubiquitination of FANCD2). In fact, there may be one large complex containing all of the subunits of complexes 1 and 2.

We will now give a brief overview of the entire Fanconi pathway and recently identified components before considering the structures and functions of complex 1 and complex 2 in more detail. Complex 1, the monoubiquitin E3 ligase complex, or core complex, consists of

Table 1. FA complementation groups and FA genes

FA complementation group	FA gene	Approximate frequency in FA patients (%)	Chromosomal location	Protein product (kDa)	Reference
A	<i>FANCA</i>	60%	16q24.3	163	Lo Ten Foe et al. 1996
B	<i>FANCB</i>	Rare	Xp22.31	95	Meetei et al. 2004
C	<i>FANCC</i>	15%	9q22.3	63	Strathdee et al. 1992b
D1	<i>BRCA2</i>	5%	13q12.3	380	Howlett et al. 2002
D2	<i>FANCD2</i>	5%	3p25.3	155, 162	Timmers et al. 2001
E	<i>FANCE</i>	Rare	6p21.3	60	de Winter et al. 2000a
F	<i>FANCF</i>	Rare	11p15	42	de Winter et al. 2000b
G	<i>FANCG</i>	10%	9p13	68	de Winter et al. 1998
I	Unknown	Rare	Unknown	Unknown	Unknown
J	<i>BRIP1</i>	Rare	17q23.2	130	Levitus et al. 2005
L	<i>FANCL</i>	Rare	2p16	52	Meetei et al. 2003a
M	<i>FANCM</i>	Rare	14q21.2	250	Meetei et al. 2005

8 of the 11 identified FA proteins (A, B, C, E, F, G, L, M). The putative catalytic element of this multisubunit complex is the L protein. The L subunit contains a PHD domain, and has homology to other E3 ubiquitin ligases (Meetei et al. 2003a). Complex 1 is required for the monoubiquitination of FANCD2 on Lys 561 during S phase of the normal cell cycle. Complex 1 is also essential for the inducible monoubiquitination of FANCD2 following cellular exposure to DNA damaging agents, such as MMC, ultraviolet (UV), or ionizing radiation (IR) and deoxynucleotide depletion due to hydroxyurea (Garcia-Higuera et al. 2001; Gregory et al. 2003). Following monoubiquitination, FANCD2 relocates from a soluble nuclear compartment to the chromatin fraction (Wang et al. 2004) where it colocalizes in nuclear foci with other DNA damage response proteins, including FANCD1/BRCA2 (Hussain et al. 2004; Wang et al. 2004) BRCA1 (Garcia-Higuera et al. 2001), RAD51 (Taniguchi et al. 2002a; Hussain et al. 2004), Proliferating Cell Nuclear Antigen (PCNA) (Hussain et al. 2004), and Nijmegen Breakage Syndrome (NBS1) (Nakanishi et al. 2002). Following relocation to chromatin, monoubiquitinated FANCD2 (FANCD2-Ub) promotes chromatin loading of the FANCD1/BRCA2 protein, forming a second complex (complex 2), where it may facilitate the formation of nuclear foci containing the homologous recombination (HR) DNA repair protein RAD51 (Wang et al. 2004). FA cells that fail to form either complex 1 or complex 2 are hypersensitive to cross-linking agents such as MMC and exhibit the characteristic broken and radial chromosome forms of FA.

Recently identified components of the FA pathway

Three additional FA pathway components have recently been reported. Traditional approaches, based on complementation studies of cells derived from FA patients, have identified the groups FANCI and FANCF (Levitus et al. 2004). Experimental approaches involving the knockout of genes in chicken and human cell lines have identified FANCM (Meetei et al. 2005; Mosedale et al. 2005). We

will now consider where these new components may function within the context of the basic FA pathway.

FANCI, an unknown component of complex 1?

FANCI cells express the multisubunit FA complex (A/B/C/E/F/G/L/M complex), but fail to monoubiquitinate FANCD2 (Levitus et al. 2004). Accordingly, the FANCI protein may be another loosely associated protein of complex 1, such as an E2 ubiquitin conjugating protein, which is not required for complex assembly but rather is required for E3-mediated monoubiquitination of FANCD2. To date, however, the *FANCI* gene has not been cloned.

FANCM is the mammalian ortholog of Hef

Very recently, two groups have identified the human ortholog of helicase-associated endonuclease for forked structured DNA (Hef) as a component of FA complex 1. Hef was originally described in archaeobacteria as a protein that could process blocked DNA replication forks into double-strand DNA breaks (Komori et al. 2002). Meetei et al. (2005) identified the human ortholog of Hef as a 250-kDa component of complex 1 using coimmunoprecipitation experiments. Small interfering RNA (siRNA)-mediated depletion of this protein in HeLa cells resulted in a failure to monoubiquitinate FANCD2, confirming a functional role in complex 1. Genotyping of an individual with previously unclassified Fanconi Anemia identified a nonsense mutation in the *Hef* gene on one allele and a partial deletion of the gene on the other. They concluded that mutation in *Hef* represents a new FA complementation group, FANCM. However, it is important to note that FA-M cells have not yet been complemented with a wild-type *FANCM* gene construct. This may be due to difficulty in ectopically expressing the 250-kDa protein (Meetei et al. 2005).

In a separate study, Mosedale et al. (2005) used knockout studies to characterize the function of the *Hef* ortholog in DT40 chicken cells. They noted that *Hef*-knockout

cells are highly sensitive to MMC and demonstrate chromosomal instability. When Hef was knocked out of *FANCC* disrupted cells, no further sensitivity was noted, indicating that Hef functions within the FA pathway. Consistent with this hypothesis, Hef coimmunoprecipitated with labeled *FANCF* and *FANCC*, suggesting that it is a component of complex 1. Furthermore, Hef was required for the relocalization of complex 1 to chromatin following DNA damage. They also noted that Hef was required for DNA damage-induced *FANCD2* monoubiquitination, indicating that it has a role in the monoubiquitin ligase activity of complex 1 (Mosedale et al. 2005). Interestingly, the *FANCM*-deficient DT40 cells were defective in the HR mediated repair of DNA cross-links due to MMC but demonstrated normal HR repair of double-strand breaks (DSBs). This observation suggests some specificity for the FA pathway in the processing of DNA cross-links as opposed to other forms of DNA damage and could explain the specific high sensitivity to cross-linking agents seen in cell lines derived from FA patients.

In bacterial cells, Hef has both endonuclease and helicase domains that function together to unwind and cleave synthetic complexes that mimic stalled replication forks (Komori et al. 2002). Similarly, the N-terminal region of human *FANCM* is homologous to the DEAH-box ATP-dependent helicase family, whereas the C terminus is homologous to the endonuclease region of the nucleotide excision repair protein ERCC4. In contrast to bacterial Hef, the endonuclease domain of *FANCM* appears to be degenerate and nonfunctional (Meetei et al. 2005). Indeed, mutation of the endonuclease domain of *FANCM* in DT40 cells does not markedly affect cross-linker sensitivity (Mosedale et al. 2005). However, *FANCM* does demonstrate an in vitro DNA-dependent ATPase function with an affinity for single-strand and forked structures that is lost when the helicase domain is mutated (Meetei et al. 2005; Mosedale et al. 2005). Mutation of the helicase domain also results in marked cisplatin sensitivity in DT40 cells. Importantly, no in vitro helicase activity has been demonstrated for *FANCM*. Rather, *FANCM* has been shown to function as a DNA translocase in an in vitro triple-helix displacement assay (Meetei et al. 2005). This observation supports a model where *FANCM* moves the FA complex along DNA, perhaps in association with a replication fork. *FANCM* may be specifically involved in the detection of a cross-link at a stalled replication fork and may then respond by activating the rest of the FA pathway. From the recent studies it appears that *FANCM* function may be ATP dependent, and it would be interesting in the future to investigate if mutation of the ATPase region of this protein affects the chromatin association of complex 1 and monoubiquitination of *FANCD2* following DNA cross-linker treatment.

FANCF is the DNA helicase *BRIP1*

Three groups have recently reported that *FANCF* is the BRCA1-Interacting Protein C-terminal helicase *BRIP1*

(also known as BACH1). Interestingly, in common with *BRCA1* and *FANCD1/BRCA2*, heterozygous mutation of *BRIP1* predisposes to early onset breast cancer, further supporting a role for the FA pathway in breast cancer development (Cantor et al. 2001).

Levitus et al. (2005) used positional cloning and a microcell-mediated chromosome complementation approach to study eight cell lines derived from FA-J individuals. They identified eight different mutations affecting the helicase domain of *BRIP1*, of which five were predicted to result in a protein truncation and the remainder a full-length protein with an altered amino acid residue. Three patients were homozygous for the same mutation whereas five carried a different mutation on each allele. Complementation of *FANCF* mutant cells was achieved using part of chromosome 17 but not specifically with expression of a wild-type *BRIP1* gene or cDNA construct.

Similarly, Levran et al. (2005) used genetic mapping to initially study 5 patients assigned to complementation group FA-J and then a further 18 unclassified FA patients. They also identified homozygous mutations in the *BRIP1* gene in FA-J patients and reported an additional two truncating mutations, an insertion mutation and a missense mutation predicted to disrupt the helicase domain of the *BRIP1* protein.

Two other groups also recently identified *BRIP1* as *FANCF* by using knockout approaches in cell lines. Litman et al. (2005) used siRNA to silence *BRIP1* expression in MCF7 human breast cancer cells whereas Bridge et al. (2005) used a *BRIP1* knockout approach in chicken DT40 cells. Both studies reported that abrogation of *BRIP1* expression resulted in a FA cellular phenotype with profound cross-linker sensitivity and an accumulation in the G2/M phase of the cell cycle following DNA damage. Litman et al. also demonstrated a truncating mutation of *BRIP1* in the FA-J AG656 cell line and showed that lentiviral-mediated expression of *BRIP1* in this cell line corrected the G2/M cell cycle abnormality. Interestingly these papers reported differing results as regards the role for *BRIP1* in HR. Litman et al. found a defect in the HR of a I-SceI induced DSB in the *BRIP1*-deficient MCF7 cells whereas Bridge et al. reported normal repair of the same lesion in the *BRIP1*-deficient DT40 cells. The reason for these differing results is unclear but may relate to differences between human and chicken cells. Indeed, chicken *BRIP1* is structurally different from its human counterpart. It is only 54% homologous to human *BRIP1* and does not contain the BRCT-binding consensus sequence (Bridge et al. 2005). This sequence appears to be required for the response to DNA damage in mammalian cells by mediating an association with *BRCA1* (Yu et al. 2003).

Although the function of *BRIP1* is unclear, it seems likely that it has a role in the processing of intermediate DNA structures during repair. *BRIP1*, in common with Bloom helicase (BLM), is a member of the recQ DEAH helicase family. However, BLM in contrast to *BRIP1* has been reported to suppress sister chromatid exchange (SCE) in HR (Onclercq-Delic et al. 2003). *BRIP1* demon-

strates an in vitro 5'–3' DNA unwinding activity of forked DNA duplexes whereas BLM demonstrates a 3'–5' helicase activity (Karow et al. 1997; Gupta et al. 2005). BRIP1, in common with BLM, also associates with DNA substrates mimicking Holliday Junctions (Litman et al. 2005) although, in contrast to BLM, no unwinding of these structures has been demonstrated (Gupta et al. 2005). Therefore, it has been hypothesized that BRIP1 may promote HR by blocking the access of BLM to intermediate DNA structures or by providing a scaffold for other HR proteins (Litman et al. 2005). Although the role for BACH-associated helicase activity remains unclear, its importance is demonstrated by the observation that disease-related mutations all disrupt the helicase domain (Levitus et al. 2005; Levran et al. 2005). Moreover, specific mutation of the helicase domain of BRIP1 in chicken DT40 cells results in hypersensitivity to cisplatin (Bridge et al. 2005).

Another question that both these papers raise is the role of BRCA1 in the FA pathway. BRIP1 is phosphorylated and associates with the BRCA1 C Terminus (BRCT) motifs of BRCA1 during S phase of the cell cycle (Rodriguez et al. 2003; Yu et al. 2003). The association between BRCA1 and BRIP1 has been reported to be essential for DNA repair and cell cycle checkpoint functions following radiation-mediated DNA damage (Cantor et al. 2001; Yu et al. 2003). However, Litman et al. (2005) reported that BRIP1 was not required for RAD51 foci formation after hydroxyurea treatment whereas BRCA1 was essential, indicating that these proteins may have some distinct functions. Bridge et al. (2005) also reported that BRIP1 functioned independently of BRCA1 in the DT40 cell line. One possible explanation for these discrepancies is that the role of BRIP1 in the response to replication fork arrest is distinct from that in radiation damage repair and independent of BRCA1. Indeed, *BRIP1* knockout DT40 cells are not very sensitive to radiation damage but are hypersensitive to cross-linking agents (Bridge et al. 2005). It is also important to note that the chicken ortholog of *BRCA1* may not function identically to the human counterpart. Chicken BRCA1 protein is

only 33% homologous to human BRCA1 (Orelli et al. 2001). Abrogation of chicken *BRCA1* does not result in hypersensitivity to cisplatin or to radiation damage (Bridge et al. 2005) unlike mammalian *BRCA1* in which loss of function has been reported to result in marked sensitivity to both forms of DNA damage (Xia and Powell 2002; Quinn et al. 2003; Kennedy et al. 2004). Moreover, the observations that functional BRCA1 is required for DNA damage-induced FANCD2 foci (Garcia-Higuera et al. 2001) and that loss of *BRCA1* function results in marked cross-linker sensitivity, chromosomal breakage, and a defect in HR (Moynahan et al. 2001a) further support a model in which *BRCA1*, in common with *BRIP1*, has a role in the FA pathway.

At present the role of BRIP1 in the FA pathway is uncertain. FA-J cells, in common with FA-D1 (*BRCA2* mutant) cells, express an intact FA complex 1 and exhibit normal to elevated levels of FANCD2-Ub (Levitus et al. 2004; Litman et al. 2005). These observations suggest that BRIP1 functions independently or downstream of FANCD2, perhaps as another subunit of the FANCD2-Ub/BRCA2 chromatin complex (complex 2 in Fig. 1). It is clear that future studies are required to elucidate the role of BRIP1 in the HR of DNA cross-linking lesions and answer if there is a requirement for the interaction with BRCA1. It is also important to note that no study to date has reported the correction of chromosomal breakage in FA-J cells through the exogenous expression of wild-type *BRIP1*. The generation of isogenic mutant and fully corrected cell lines would be expected to help answer many of the questions about the role of *BRIP1* in the FA pathway.

The structure of the FA complex 1

At least eight of the FA proteins (subunits A, B, C, E, F, G, L, M) interact in a large nuclear complex. This complex is shown schematically in Figure 2. While no crystal structures are available for subunits of the complex, several interactions are evident from yeast two-hybrid and coimmunoprecipitation studies.

Figure 1. Schematic interaction of the FA pathway. Protein complex 1 contains the A, B, C, E, F, G, L, M, and possibly I subunits. Protein complex 2 contains FANCD2-Ub, BRCA2, and possibly FANCI. During S phase, when a replication fork encounters a DNA cross-link, the FA complex (complex 1) is activated. This activation leads to the monoubiquitination of FANCD2, which is then targeted to chromatin containing the cross-link. FANCD2-Ub then interacts with BRCA2 in complex 2, leading to repair of the cross-link possibly through HR and TLS. FANCD2 is deubiquitinated by USP1, thereby inactivating the pathway.

Figure 2. Schematic structure of the Fanconi Anemia E3 ligase complex (complex 1). The core complex contains subunits A, B, C, E, F, G, L, M, and possibly I. According to this model, ubiquitin is transferred from an E1 to an E2 (unknown for the FA complex) to FANCD2. The FANCD2 substrate may have direct binding interactions with FANCL and FANCE, and these interactions may be mediated, at least in part, by ATR-dependent phosphorylations on the FANCD2 protein. Importantly, recent studies indicate that complex 1 is associated with chromatin and may function as a sensor of DNA damage as well as an E3 ligase.

The FANCA and FANCG subunits interact directly, and these subunits are dependent on each other for stability. Absence of the FANCA protein shortens the half-life of the FANCG protein (Garcia-Higuera et al. 2000). Molecular studies support interactions between FANCG and FANCF (Garcia-Higuera et al. 2000), between FANCE and FANCC (Pace et al. 2002; Taniguchi and D'Andrea 2002), and between FANCB and FANCL subunits (Meetei et al. 2004). The FANCE protein is required for the efficient transport of the FANCC protein to the nucleus (Pace et al. 2002; Taniguchi and D'Andrea 2002). Interestingly, yeast two-hybrid studies have also indicated that the N terminus of FANCE interacts directly with FANCD2 (Gordon and Buchwald 2003), implying that complex 1 may associate with FANCD2 through FANCE, thereby facilitating FANCD2 monoubiquitination. The newly cloned FANCM, in common with other FA proteins, has been shown to be required for the stability of complex 1 and coimmunoprecipitates with FANCC and FANCF, although a direct interaction with these proteins has not been shown (Meetei et al. 2005; Mosedale et al. 2005).

Coimmunoprecipitation experiments support the presence of subcomplexes (for example, the FANCA/FANCG and FANCE/FANCF/FANCG/FANCC subcomplexes), which may preform and translocate intracellularly before the assembly of the complete complex. Specific domains of these subunits are likely to play a role in subunit assembly, such as the tetratricopeptide repeat (TPR) domains of the FANCG subunit (de Winter et al. 1998) and the WD40 domains of the FANCL sub-

unit (Meetei et al. 2003a). Phosphorylation of some of the FA subunits also appears to contribute to complex 1 assembly (Kupfer et al. 1999).

The FANCL protein has a C-terminal RING-finger-like plant domain (PHD), believed to be directly involved in the catalytic activity of the complex. The PHD domain may interact directly with an E2 protein of the FA pathway. Interestingly, a truncated form of FANCL, lacking the C-terminal PHD domain, still binds and stabilizes the FA complex, through its three WD40 domains, but does not complement the FANCD2 monoubiquitination defect in FANCL cells (A. Gurtan and A.D. D'Andrea, unpubl.). WD40 domains of FANCL may be required for its interaction with phosphorylated residues on other FA complex subunits or perhaps with phosphorylated residues on the FANCD2 substrate. It has previously been reported that the WD40 domains of other E3 ligases are important in recruiting specific substrates to the ligase complex for ubiquitination (Orlicky et al. 2003).

While the FA complex is the putative E3 ligase for FANCD2, the *in vitro* reconstitution of this ligase reaction has not been successful to date. In fact, the only E3 ligase that has been demonstrated to monoubiquitinate FANCD2 *in vitro* is the BRCA1/BARD1 complex (Vandenberg et al. 2003). Additional subunits of the FA complex, such as the putative FANCI protein, may be required for reconstitution of the FANCL E3 ligase activity *in vitro*. However, the FANCL subunit does undergo autoubiquitination *in vitro*, and its PHD domain is required for this activity (Meetei et al. 2003a).

As will be discussed later, Bloom DNA helicase and topoisomerase III have also been reported to associate with complex 1, which, along with recent identification of FANCM as a DNA helicase, suggests that this complex has a direct role in DNA processing as well as having a monoubiquitin E3 ligase activity.

In conclusion, complex 1 has an E3 ubiquitin ligase activity directed toward FANCD2 and a DNA processing activity of uncertain purpose. Important questions remain about complex 1. First, is complex 1 associated with the advancing replication fork and does it bind to helix-distorting lesions, thereby acting as a sensor of damage? Second, do other specific subunits of complex 1, in addition to FANCM, bind directly to DNA? Third, while direct interactions among the subunits of complex have been defined by coimmunoprecipitation and yeast two-hybrid experiments, what are the precise crystallographic interactions?

The structure of FA complex 2

The downstream function of the FA pathway is largely unknown. Accumulating evidence suggests a major function of complex 1 is to generate monoubiquitinated FANCD2 (FANCD2-Ub), required for the assembly of the FANCD2-Ub/BRCA2 chromatin complex (complex 2). Following monoubiquitination, FANCD2 relocates to DNA damage-induced S-phase foci where it associates with the DNA repair proteins BRCA2/FANCD1 (Wang

et al. 2004), BRCA1 (Garcia-Higuera et al. 2001), NBS1 (Nakanishi et al. 2002), RAD51 (Hussain et al. 2004), and PCNA (Howlett et al. 2005). The formation of these DNA damage-induced FANCD2 foci is dependent on the presence of functional BRCA1 (Garcia-Higuera et al. 2001), thereby linking this DNA repair protein to the FA pathway. As discussed earlier, *BRIP1* (*FANCF*) mutant cells demonstrate normal FANCD2 monoubiquitination, indicating that it does not form part of complex 1. Furthermore, BRIP1, in common with FANCD2, localizes to DNA damage-induced nuclear foci along with BRCA1 (Cantor et al. 2001), suggesting that it may function as part of complex 2. A diagrammatic representation of FA complex 2 is shown in Figure 1.

Perhaps the simplest model proposes that FA complex 2 contributes to the stabilization of a stalled DNA replication fork. Indeed BRCA2 (*FANCD1*) has been demonstrated to be required for the stabilization of the stalled replication fork following treatment with hydroxyurea (Lomonosov et al. 2003). By stabilizing the fork, the complex may facilitate DNA repair, and may be required for the recruitment of repair proteins. Accordingly, it will be interesting to determine whether disruptions of FANCD2 or BRIP1/*FANCF* also result in destabilization of the replication fork.

In conclusion, complex 2 may contain a helicase activity (BRIP1) and appears to play a more downstream role in DNA repair. Important data about this complex still remain to be elucidated. First, as will be discussed further later, current data indicate that the FA pathway interacts with the process of HR repair and translesion synthesis (TLS) (Niedzwiedz et al. 2004). How complex 2 engages in these two processes remains unclear. Second, whether complex 2 has a direct function in cell cycle checkpoint activation remains unknown. Third, the molecular interactions among the protein subunits of complex 2 remain to be elucidated. Finally, it is not clear whether BRCA1 and its associated E3 ubiquitin ligase activity also play a role in the function of this complex.

Activation of the FA pathway

Now that we have described the components of the basic FA pathway, we will address how the pathway is activated following DNA damage. Several findings indicate that the Fanconi pathway is activated and functions primarily in the S phase of the cell cycle. First, cell cycle synchronization studies reveal that FANCD2 monoubiquitination occurs predominantly in S phase. FANCD2 is monoubiquitinated at the G1/S boundary, remains monoubiquitinated throughout S phase, and becomes deubiquitinated at the end of S phase as the synchronized cell population enters G2 (Taniguchi et al. 2002a). Second, during S phase, monoubiquitinated FANCD2 protein specifically colocalizes with replication foci containing BRCA1, RAD51 (Taniguchi et al. 2002a; Hussain et al. 2004), and PCNA proteins (Howlett et al. 2005). These foci are believed to represent sites of homologous

repair. Third, the FA complex 1 associates with chromatin during S phase, and is released in mitosis (Mi and Kupfer 2005).

Recent studies indicate that the FA pathway is activated during S phase in response to DNA replication fork arrest. Replication fork arrest leads to the local generation of single-strand DNA, perhaps through helicase unwinding of the DNA duplex. Single-strand DNA binds RPA (Replication Protein A) and then ATR (Ataxia Telangiectasia and RAD3-related protein) and ATRIP (ATR-Interacting Protein). After recruitment to the stalled fork, ATR phosphorylates multiple substrates, leading to additional cell cycle checkpoint and DNA repair responses (Zou and Elledge 2003). One principal substrate of ATR is CHK1 (Checkpoint Kinase 1). Recent studies implicate ATR/CHK1 in the activation of the FA pathway. First, agents that activate ATR kinase, such as hydroxyurea, cross-linkers, or ultraviolet light are potent activators of FANCD2 monoubiquitination (Gregory et al. 2003). Second, cells from patients with Seckel syndrome express a mutant ATR and fail to monoubiquitinate FANCD2 following DNA damage. Similarly, siRNA-mediated silencing of ATR expression abrogates FANCD2 monoubiquitination (Andreassen et al. 2004). Third, knockdown of ATR also results in a chromosome breakage phenotype and cellular hypersensitivity to MMC, consistent with an upstream role for ATR in the FA pathway (Andreassen et al. 2004). Moreover, disruption of the CHK1 kinase either by siRNA knockdown or by specific inhibitors of CHK1 kinase leads to DNA cross-linker sensitivity and a disruption of the FA pathway (X. Wang and A.D. D'Andrea, unpubl.). Fourth, the recently identified FANCM protein is hyperphosphorylated following DNA damage. This protein has multiple ATR consensus sites, although the ATR-mediated phosphorylation of these sites have yet to be investigated (Meetei et al. 2005).

Taken together, these results support a model in which ATR and CHK1, in response to DNA replication fork stalling, modulate the FA pathway. This may occur through the direct phosphorylation of one or more subunits of complex 1. For example, phosphorylation of FANCM may promote the DNA translocation of complex 1 and facilitate the identification of DNA cross-links, leading to subsequent activation of the rest of the FA pathway, which in turn may be involved in the processing of the lesion that has given rise to the failure in DNA replication.

Functions of the FA pathway

Despite extensive research into this area, the specific functions of the FA pathway following activation by DNA replication fork arrest are uncertain. However, recent studies suggest that it may function to coordinate several aspects of DNA damage response including (1) direct enzymatic DNA processing, (2) recruitment of DNA repair proteins involved in HR and TLS, and (3) cell cycle checkpoint activation (Fig. 3).

Figure 3. A schematic diagram depicting how the FA pathway may function to coordinate the cellular response to replication fork arrest following DNA damage. ATR is activated in response to replication fork arrest and in turn may activate the FA pathway, which detects the DNA lesion and coordinates its repair.

Enzymatic DNA processing

As discussed earlier, the most recently identified FA proteins (J and M) have DNA helicase domains (Levitov et al. 2005; Meetei et al. 2005). BLM and topoisomerase III have also been found to associate with FA complex 1 (Meetei et al. 2003b). BLM, topoisomerase III, and replication protein A associate with constituents of the FA complex 1 (the BRAF complex) (Meetei et al. 2003b). These proteins have been shown to cooperate in DNA unwinding and have been implicated in the response to replication fork arrest (Chan et al. 1987; Brosh Jr. et al. 2000; Wu and Hickson 2002). Mutation of *BLM* in common with other FA genes results in chromosomal breakage (Risinger and Groden 2004), although it is important to note that *BLM*-deficient cells also exhibit increased SCE, a phenotype not observed in FA cells.

FA complex 2 colocalizes with NBS1, a component of the MRE11/RAD50/NBS1 (MRN) complex that has 3'-to-5' exonuclease activity and is required for DNA processing prior to repair through HR (Nakanishi et al. 2002; Kobayashi et al. 2004). Loss of function of the FA pathway has been associated with a failure of the MRN complex to form at sites of DNA damage (Pichierri et al. 2002).

Overall, these observations linking the FA pathway to DNA processing suggest that the FA proteins not only function in DNA damage signaling pathway but may also have active roles in the detection and processing of stalled replication forks. Consequently, loss of the FA pathway may lead to chromosomal instability through the failure to process DNA repair intermediates.

Recruitment of DNA repair proteins

Homologous recombination (HR) HR is a process by which double-strand DNA breaks are repaired through

the alignment of homologous sequences of DNA and occurs primarily during the late S to M phase of the cell cycle. This repair pathway requires the 3'-5' exonuclease activity of the RAD50, MRE11, and NBS1 complex to expose the 3' ends on either side of the DSB, a process that may also require BRCA1 (Zhong et al. 1999). The break can then be repaired by one of two main pathways. Either single-strand annealing, a potentially mutagenic process that uses regions of homology between the complementary strands to align the broken ends of DNA, or strand invasion, a RAD51- and BRCA2-dependent process that typically uses either a homologous sequence in adjacent DNA or the sister chromatid as a template to repair the break. This latter process may result in a SCE of genetic material (Wyman et al. 2004).

During S phase, DNA cross-links result in a replication fork arrest. In the process of repair, the cross-links are excised by an endonuclease leading to the generation of DSB intermediates (Dronkert and Kanaar 2001; Rothfuss and Grompe 2004). The DSB can then be repaired by HR. Following treatment with cross-linkers, DSB formation has been reported to precede FANCD2 monoubiquitination. FANCD2-Ub then colocalizes with Histone 2AX S-phase foci, a marker of DSBs, indicating a role for the FA pathway in the repair of this type of DNA damage. Accordingly, disruption of the FA pathway results in persistence of DNA breakage (Rothfuss and Grompe 2004). It is interesting to note that although XPF/ERCC1 has been reported to colocalize with FANCA, suggesting it may function in the FA pathway (Sridharan et al. 2003), XPF/ERCC1 mutant cells demonstrate normal FANCD2 monoubiquitination (R.D. Kennedy and A.D. D'Andrea, unpubl.). This implies that another endonuclease such as the Mus81-Eme1 complex (Abraham et al. 2003) is, at least partly, involved in the excision of DNA cross-links upstream in the FA pathway.

Monoubiquitinated FANCD2 associates with BRCA1 (Garcia-Higuera et al. 2001), BRCA2 (Hussain et al. 2004; Wang et al. 2004), RAD51 (Hussain et al. 2004), PCNA (Howlett et al. 2005), and NBS1 (Nakanishi et al. 2002) and is required for the relocalization of BRCA2 to chromatin and the subsequent formation of RAD51 foci (Hussain et al. 2004; Wang et al. 2004). Although the purpose of FANCD2 monoubiquitination remains unknown, it seems likely that it has a role in HR as BRCA1, BRCA2, RAD51, and NBS1 have all been implicated in this process (Moynahan et al. 1999; Tutt et al. 2001; Kobayashi et al. 2004). In support of this hypothesis, cell lines deficient in the FA pathway have been reported to have defective HR (Donahue et al. 2003; Yamamoto et al. 2003; Niedzwiedz et al. 2004; Nakanishi et al. 2005). In general, disruption of upstream FA proteins, such as FANCA (Nakanishi et al. 2005) and FANCG (Yamamoto et al. 2003) result in milder HR defects than disruption of BRCA2/FANCD1 (Moynahan et al. 2001b), suggesting that the FA chromatin-associated complex 2 is largely responsible for the DNA repair functions of the FA pathway. Interestingly, recent evidence suggests that DNA cross-links may be preferentially repaired by the single-strand annealing subset of HR (Jonnalagadda et al. 2005).

Murine cells deficient in components of complex 1 or FANCD2 demonstrate abnormal single-strand annealing following cross-linker damage, suggesting a specific role for the FA pathway in this subset of HR (Nakanishi et al. 2005; Yang et al. 2005).

Translesion synthesis (TLS) Another proposed role for the FA pathway is TLS. This process can result in point mutations due to the misincorporation of nucleotides. TLS may offer an alternative to HR in order to re-establish replication forks or may work in conjunction with HR in the resolution of certain types of DNA damage (Niedzwiedz et al. 2004). The best characterized DNA damage resulting in TLS is the ultraviolet-induced cyclobutane pyrimidine dimer (CPD), although TLS is also likely to bypass DNA adducts such as those produced by cross-linking agents (Yamashita et al. 2002). CPD damage is either repaired by nucleotide excision repair (NER) or bypassed by TLS (Friedberg et al. 2002; Prakash and Prakash 2002). At the beginning of the process of TLS, a normal replicative DNA polymerase, such as Pol δ , encounters a CPD and is displaced by a TLS polymerase (Friedberg et al. 2005). The TLS polymerase can replicate past CPD damage with higher fidelity and efficiency than a replicative polymerase (Yu et al. 2001). Evidence for the role of the FA pathway in TLS comes from experiments in the DT40 chicken cell line where loss of the TLS proteins Rev1 and Rev3 along with FANCC are epistatic as regards DNA cross-linker sensitivity. Rev1 and monoubiquitinated FANCD2 are also observed to colocalize in nuclear foci following replication fork arrest (Niedzwiedz et al. 2004). Loss of the FA pathway results in a hypomutable phenotype following cross-linker treatment, further suggesting a relationship between the FA and TLS pathway (Papadopoulos et al. 1990a,b). Furthermore, Rev1 deficient DT40 cells, in

common with FA cells, demonstrate hypersensitivity to cisplatin (Ross et al. 2005). Finally, as will be discussed later, recent evidence suggests that the FA pathway and TLS are terminated by the same deubiquitinating enzyme, USP1, further indicating their coordinate regulation (T. Huang and A.D. D'Andrea, unpubl.).

One hypothesis, which may link the HR and TLS functions of the FA pathway, is the requirement for both processes in the repair of DNA cross-links. These lesions are unusual in that they require the excision of adducts on both strands of DNA and the subsequent repair of the resultant DSB in S phase (Rothfuss and Grompe 2004). The FA pathway may function to coordinate the excision and bypass of DNA adducts with the HR of DSBs. Consistent with this model, components of the FA pathway, HR, and TLS have all been shown to be required for the repair of cross-links in chicken DT40 cells (Niedzwiedz et al. 2004). Figure 4 demonstrates a simplified version of the model recently proposed by Niedzwiedz et al.

Niedzwiedz et al. (2004) also reported some interesting data regarding the role of the FA pathway and TLS in the response to endogenous DNA damage. Unlike cross-link repair, in which TLS and HR appear to cooperate, the FA pathway promotes either HR or TLS independently in the repair of abasic sites (Niedzwiedz et al. 2004). Therefore, loss of the FA pathway may be predicted to result in a failure to repair endogenous DNA damage efficiently, thereby promoting chromosomal instability.

In contrast to the *Rev1* and *Rev3* knockout studies, *Rad18*, another TLS protein, has been reported to function independently of FANCC in DT40 cells. The Rad6/*Rad18* complex mediates PCNA monoubiquitination, thereby facilitating recruitment of a TLS polymerase. The combination of *Rad18* and *FANCC* knockout results in greater cisplatin sensitivity than either knocked

Figure 4. A simplified model of FA-mediated cross-link repair that involves NER, TLS, and HR. (1) The replication fork arrests at a DNA cross-link and is detected by FA complex 1. FA complex stabilizes the arrested fork. (2) NER excises one side of the cross-link leaving a single-strand DNA break on one strand and a monoadduct on the opposite strand. (3) FA recruits TLS to bypass the remaining monoadduct. (4) NER excises the monoadduct. (5) FA-mediated HR of double-strand DNA break. (6) Intermediate DNA crossover structures are resolved and the replication fork is re-established, a process that may require FA pathway-associated DNA helicases.

out independently (Hirano et al. 2005). These data suggest that FA pathway-mediated TLS may be Rad18 independent. Indeed, Rad18-independent TLS has been reported in vertebrate cells (Okada et al. 2002).

Cell cycle regulation

FA cells characteristically demonstrate an accumulation of 4N DNA content either spontaneously or following treatment with DNA cross-linkers (Kubbies et al. 1985). Indeed, this consistent observation has been proposed as a diagnostic test for FA (Miglierina et al. 1991). One hypothesis to explain this phenotype has been that FA cells fail to arrest in S phase due to DNA damage and accumulate at the G2/M damage checkpoint (Centurion et al. 2000; Sala-Trepat et al. 2000). Other investigators have proposed that FA cells arrest in late S phase due to a failure to remove DNA cross-links (Akkari et al. 2001). At present, the function of the FA pathway in cell cycle regulation remains uncertain, largely due to the different methodologies and cell line models used to study this question. Published evidence suggests that the FA pathway may have roles in the DNA replication, intra-S-phase, and G2/M DNA damage checkpoints.

The DNA replication checkpoint is activated during S phase in response to stalled DNA replication and primarily involves the ATR-mediated phosphorylation of DNA damage response kinases such as CHK1. It functions to allow the stabilization and repair of the stalled replication fork or the restoration of a depleted deoxynucleotide pool. It also functions to stop the firing of further DNA replication origins, preventing the formation of new replication forks until the existing damage has been repaired (Bartek et al. 2004). A role for the FA pathway in the replication checkpoint is supported by the observations that FANCD2 monoubiquitination is dependent on ATR activation and that constituents of complex 1 (the E3 ligase complex) and FANCD2 have both been shown to be required for S-phase cell cycle arrest following cross-link damage (Centurion et al. 2000; Sala-Trepat et al. 2000; Pichierri and Rosselli 2004).

In addition to the replication checkpoint, there is evidence that the FA pathway may function in a second S-phase checkpoint, the intra-S-phase checkpoint. This checkpoint is predominantly activated by the ataxia-telangiectasia-mutated (ATM) kinase and the ATM substrate CHK2 in response to double-strand DNA breakage and is not dependent on replication fork arrest (Bartek et al. 2004). Cells derived from FA patients replicate DNA in the presence of radiation damage, a feature of a deficient intra-S-phase checkpoint. ATM-mediated phosphorylation of FANCD2 at Ser 222, a process that also depends on NBS1, has been shown to be required for this checkpoint.

Indeed, recent studies suggest a direct interaction between the C terminus of FANCD2 and ATM (S. Margosian and A.D. D'Andrea, unpubl.). Interestingly, activation of the intra-S-checkpoint is independent of the monoubiquitination of FANCD2 (Taniguchi et al. 2002b).

Recent data also suggest that the FA pathway has a role in maintaining the G2/M boundary DNA damage checkpoint, which exists to prevent progression into mitosis prior to the repair of abnormally replicated DNA. This checkpoint is activated by either ATM or ATR and their downstream kinase targets depending on the type of DNA damage (Liu et al. 2000; Shiloh 2001; Helt et al. 2005). Murine cells deficient in *FANCC* or *FANCD2* undergo an abnormal progression into mitosis following DNA damage when compared with wild-type cells (Freie et al. 2004). Interestingly, loss of *BRIP1* (*FANCF*) function also results in a defect in the G2/M checkpoint, suggesting that this protein may function in a common pathway with the other FA proteins in G2/M checkpoint regulation (Yu et al. 2003).

Turning off the FA pathway: the FANCD2 Dub

Inappropriate activation of the FA pathway in undamaged cells may lead to harm by promoting mutagenic TLS. Consequently cells may have developed mechanisms of shutting off the pathway when it is not needed. The Deubiquitinating enzyme (Dub), USP1, has recently been identified as a critical enzyme that regulates the FA pathway. Dub enzymes are cysteine proteases that cleave peptide and isopeptide linkage immediately after the glycine-glycine sequence (i.e., the C terminus of ubiquitin) (D'Andrea and Pellman 1998; Wilkinson 2000; Amerik and Hochstrasser 2004).

The role of USP1 in the FA pathway was originally identified using a retroviral small hairpin RNA (shRNA) library (Nijman et al. 2005). Pools of shRNA molecules were used to abrogate the expression of specific Dub enzymes. ShRNA depletion of USP1 resulted in increased FANCD2-Ub levels, without promoting DNA damage. Moreover, USP1 activity peaks at the end of S phase, perhaps accounting for the observed deubiquitination of FANCD2 in late S phase.

Interestingly, USP1 has also recently been identified as the Dub for PCNA, and therefore has a role in down-regulating TLS (T. Huang and A.D. D'Andrea, unpubl.). Loss of USP1 function results in elevation and prolongation of PCNA ubiquitination. This provides further evidence for the intertwined nature of the FA pathway and TLS. It is important to note that, unlike all the other FA proteins described in Figure 1, mutation of the USP1 protein has not yet been directly linked to the pathogenesis of FA or other human disease.

Cancer risk in heterozygous carriers of FA gene mutation

Increased cancer risk is a well-recognized clinical feature of FA and has been reviewed extensively in other articles (Alter 2003; Kutler et al. 2003b). However, more recent work has shown that although heterozygous carriers of FA gene mutations do not demonstrate developmental or bone marrow abnormalities, there is a predisposition to cancer in these individuals. In particular, studies have

reported a strong association between heterozygous mutation of FA genes and susceptibility to breast or ovarian cancer. Carriers of mutations in *BRCA1* or *BRCA2/FANCD1* have an 82% lifetime risk of breast cancer and a 54% and 23% risk of ovarian cancer, respectively (King et al. 2003). Mutations in *FANCF* (*BRIP1*) have also been identified in patients with early onset breast cancer (Cantor et al. 2004) although the lifetime risk is unknown. This observation suggests that the FA pathway is important in the prevention of these female cancers and that unidentified mutations of other FA genes may account for some familial breast/ovarian cancer pedigrees not accounted for by *BRCA1* or *BRCA2/FANCD1*.

Why mutations in these components of the FA pathway specifically lead to breast and ovarian cancers is unknown. One possibility is that estrogen in some way promotes the survival of breast and ovarian cells that have undergone critical DNA damage, whereas apoptosis occurs normally in other somatic cells (Elledge and Amon 2002). Consistent with this hypothesis, oophorectomy, which diminishes serum estrogen levels, protects against breast cancer in *BRCA1* and *BRCA2/FANCD1* mutation carriers (Rebbeck et al. 2002).

Paradoxically, FA patients rarely develop breast or ovarian cancer. This may be because these patients are often hypogonadal. Accordingly, they have decreased estrogen levels, perhaps resulting in a decreased incidence of these hormonally related tumors. Also, in families carrying a *BRCA1* or *BRCA2/FANCD1* mutation, the peak age of onset of breast or ovarian cancer is in the fourth decade (Ford et al. 1994), whereas FA patients often die from complications of aplastic anemia or other cancers before this age.

In addition to breast and ovarian cancer, heterozygosity for a mutation in *BRCA2/FANCD1* predisposes to pancreatic cancer. In one study 19% of families with a history of hereditary pancreatic cancer had either a frameshift mutation or an unclassified variant of *BRCA2/FANCD1* (Hahn et al. 2003). Other FA gene mutations have also been identified in pancreatic cancer. One recent study determined that two out of 400 patients with pancreatic cancer carried a heterozygous germline mutation in *FANCC* (Couch et al. 2005). Interestingly, the pancreatic tumors from these patients exhibited loss of heterozygosity (LOH) at the *FANCC* locus, indicating that loss of *FANCC* function resulted from a mutation of one allele and deletion of the other, consistent with Knudson's two-hit hypothesis for tumor suppressor genes (Knudson Jr. 1971).

Other studies have identified human pancreatic tumor lines with biallelic loss of *FANCC* and *FANCG* (van der Heijden et al. 2004). These tumor lines were derived from individuals who were heterozygote at these loci. Taken together, these data indicate that heterozygote carriers, carrying one mutant *FANCC* or *FANCG* allele, may have increased pancreatic cancer risk, albeit at lower penetrance than in the case for a *BRCA2/FANCD1* mutation.

Other cancers that have been reported to be associated with heterozygous mutation of a FA gene include

BRCA2/FANCD1 related prostate cancer, gastric cancer, and melanoma (Liede et al. 2004), *FANCA* related acute myeloid leukemia (Condie et al. 2002; Tischkowitz et al. 2004), and *FANCC* related acute lymphoblastic leukemia (Rischewski et al. 2000).

Epigenetic disruption of the FA pathway

The human ovarian cancer cell line, 2008, fails to mono-ubiquitinate FANCD2 following DNA damage. This results from methylation of the *FANCF* promoter and a consequent loss of FANCF protein expression. Four out of 19 primary ovarian adenocarcinomas were also demonstrated to have *FANCF* methylation. Interestingly, prolonged treatment of the 2008 cell line with cisplatin resulted in restoration of FANCF expression, suggesting that correction of the FA pathway may account for acquired resistance to this treatment in a clinical setting (Taniguchi et al. 2003). Recent studies have also identified methylation of the *FANCF* promoter in 24% of ovarian granulosa cell tumors (Dhillon et al. 2004), 30% of cervical cancer (Narayan et al. 2004), 14% of squamous cell head and neck cancers, and 15% of non-small-cell lung cancers, where in the latter it correlated with a worse prognosis (Marsit et al. 2004). Epigenetic factors affecting the FA pathway may also be important for the development of sporadic acute myeloid leukemia (AML) where absent or reduced expression of the FA proteins FANCA, FANCC, FANCF, and FANCG has been reported (Xie et al. 2000). Taken together these observations imply that loss of the FA pathway is an early step in the development of some sporadic tumors or alternatively is subsequently lost in order to confer some advantage to the tumor, such as a tolerance of abnormal DNA replication.

The FA pathway and cancer treatment

The association between abnormalities in the FA pathway and cancer development may have important clinical implications as regards treatment. FA patients who are homozygous for mutation of a FA gene have a systemic DNA repair defect that results in a low tolerance for DNA-damaging chemotherapeutic agents. Accordingly chemotherapeutic agents are often given at low dosage or are avoided in favor of surgical approaches (Kutler et al. 2003a). The situation, however, is different for cancer patients who carry a heterozygous mutation in a FA gene. In this scenario the tumor contains an abnormal FA pathway and would be predicted to be more DNA damage sensitive whereas the patient's other cells, such as those in the bone marrow, contain a functional pathway and would be relatively more DNA damage resistant. Consistent with this model *BRCA2/FANCD1* mutation carriers, with breast or ovarian cancer, demonstrate a high response to DNA-damaging chemotherapeutic agents (Chappuis et al. 2002; Cass et al. 2003). It remains to be seen if malignancies associated with heterozygosity for other FA gene mutations demonstrate the same level of chemosensitivity.

Recent studies have indicated that tumor cells with a profound HR defect, such as those deficient in *BRCA1* or *BRCA2*, are highly dependent on Poly ADP Ribose Polymerase (PARP) for survival. Consequently, these tumors are hypersensitive to PARP inhibitors (Bryant et al. 2005; Farmer et al. 2005). It will be interesting to determine whether tumor cells with a disruption of the FA pathway have a comparable hypersensitivity to PARP inhibitors.

Unanswered questions

The realization that the FA pathway is intimately involved in the response to DNA damage has led to a marked growth in research in the field. Despite this increased interest, some fundamental questions still remain to be answered. In particular, it is still unknown why FA patients develop the typical syndrome of developmental abnormalities, bone marrow failure, and a predisposition to malignancy. It is also unknown why cells deficient in the FA pathway show particularly high sensitivity to cross-linking agents despite the activation of the pathway by a number of different forms of DNA damage. Here we speculate about these issues in the light of recent publications.

How do abnormalities in the FA pathway result in the FA phenotype?

Cells from FA patients have a complete disruption of the FA pathway and exhibit both spontaneous and DNA damage-inducible chromosome aberrations. Recent data implicate the tumor suppressor p53 in a mechanism that detects critical DNA damage in cells that have a disrupted FA pathway and prevents its propagation through apoptosis. When *FANCD2* is knocked out of zebrafish embryos, a proportion of the cells undergo apoptosis mediated by p53, resulting in developmental abnormalities. SiRNA-mediated silencing of *p53* in the embryo prevents this apoptotic process (Liu et al. 2003). Consistent with these observations, mice with a constitutive knockout of *FANCD2* develop to adulthood but eventually form solid tumors. Deletion of one allele of *p53* results in an earlier onset of malignancy, suggesting a failure of tumor surveillance (Houghtaling et al. 2005). Further evidence suggesting a role for p53 in the surveillance for critical DNA damage in FA cells comes from a study of squamous cell carcinomas in FA patients. This work has demonstrated an association with human papilloma virus (HPV) infection and the tumor development. HPV E6 protein is known to reduce p53 protein levels as part of the infective process, thereby preventing the activation of apoptotic pathways (Kutler et al. 2003c).

These observations suggest a model in which p53 is involved in preventing the malignant progression of FA cells (Fig. 5). Perhaps, when a FA cell has become critically damaged, p53 is involved in activating apoptosis, thereby preventing tumor formation. If this process were to happen in embryonic stem cells it may account for the

Figure 5. p53 may function to prevent the propagation of damaged DNA through apoptosis. This may lead to stem cell depletion in FA patients, causing congenital abnormalities and bone marrow failure. Loss of p53 function may predispose to cancer by allowing premalignant cells to survive.

developmental abnormalities observed. Loss of stem cells in the bone marrow may also account for the progressive anemia associated with FA. Loss of p53 or other genes involved in apoptotic function, perhaps through viral infection or FA-associated DNA breakage, may allow a cell with critical DNA damage to survive, resulting in cancer.

Why are FA cell lines specifically hypersensitive to cross-linking agents?

The FA pathway is activated by many types of genotoxic stress, including UV light, ionizing radiation, hydroxyurea, and cross-linking agents (MMC, CDDP, DEB). Surprisingly, despite the broad array of activators, FA cells are primarily hypersensitive only to cross-linking agents (Duckworth-Rysiecki and Taylor 1985; Carreau et al. 1999). What is the basis of this cross-linker specificity? Using bacterial assays, it has been reported that a single persistent DNA cross-link is enough to prevent DNA replication and cause cell death (Lawley and Phillips 1996). Given the lethality of this lesion, one hypothesis is that the FA pathway is activated by default following replication fork arrest in the possibility that a cross-link is present. Lesions that cause replication fork arrest such as UV-associated adducts or radiation-induced DNA breaks can be repaired by other processes such as NER or ATM-mediated HR and do not require a functional FA pathway. A DNA cross-link may be unique in that efficient repair requires the coordination of more than one process (Fig. 6). First the cross-link must be excised from both DNA strands. Then the resultant double-strand DNA break must be repaired, a process that may involve the coordination of HR, TLS, and cell cycle checkpoint activation by the Fanconi pathway (Fig. 4). Failure to coordinate these processes may result in a specific hypersensitivity to cross-linking agents.

Figure 6. The FA pathway may function to specifically coordinate more than one pathway following cross-linking damage, whereas IR and UV damage can be repaired through other pathways.

Conclusions

Despite the recent advances in the understanding of the FA pathway, there are many aspects to the FA pathway that remain uncertain. Examples of questions that need answers are:

- 1) Does ATR activate the FA pathway through phosphorylation of a component of complex 1?
- 2) What are the purposes of the DNA processing functions of FANCM and FANCD1, and is the ATPase function of FANCM required for activation of the FA pathway?
- 3) What is the role for BRIP1 in HR and is there a function interaction between BRCA1 and BRIP1 in mammalian cells following cross-linker damage?
- 4) What is the endonuclease that excises cross-links, upstream of the FA pathway?
- 5) How does the FA pathway participate in HR and TLS?
- 6) Why is FA complex 1, the E3 ligase for FANCD2, a large, multiunit complex?
- 7) Is the FA complex 1 or the deubiquitinating enzyme, USP1, the primary regulator of the FA pathway?
- 8) Does complex 1 have a specific affinity for cross-link-damaged DNA?
- 9) What is the E2 ubiquitin conjugating enzyme for the FA pathway?
- 10) Are there other adult cancers associated with heterozygous mutation of FA genes?
- 11) Are there cancers associated with single nuclear polymorphisms in FA genes?

The recent development of shRNA and siRNA screening libraries may help to answer some of these questions by identifying other components of the pathway (Brummelkamp and Bernards 2003).

It has now become apparent that abnormalities in the

FA pathway are not only found in childhood FA but also in the development of sporadic cancers in adults, where they may influence the response to chemotherapeutic agents. A better understanding of the pathway may allow the development of strategies to correct the pathway, thus preventing carcinogenesis in FA patients or those carrying a heterozygous mutation. Conversely, inhibitors of the pathway may be used as sensitizers to cross-linking chemotherapeutic agents in cancer treatment. It is evident that a better understanding of this complex pathway remains a priority for medical research.

Acknowledgments

We thank Hans Joenje, Weidong Wang, Kevin Hiom, and K.J. Patel for sharing data prior to publication. Funding bodies: Richard Kennedy: Susan G. Komen Breast Cancer Foundation; Alan D'Andrea: National Institutes of Health, Doris Duke Foundation.

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Genes Dev. 2005, **19**:

Access the most recent version at doi:[10.1101/gad.1370505](https://doi.org/10.1101/gad.1370505)

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