The Molecular Pathogenesis of Fanconi Anemia: Recent Progress

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Abstract

A rare genetic disease, Fanconi Anemia (FA), now attracts broader attention from cancer biologists and basic researchers in the DNA repair and ubiquitin biology fields as well as from hematologists. FA is a chromosome instability syndrome characterized by childhood-onset aplastic anemia, cancer/leukemia susceptibility, and cellular hypersensitivity to DNA crosslinking agents. Identification of eleven genes for FA has led to progress in the molecular understanding of this disease. FA proteins, including a ubiquitin ligase (FANCL), a monoubiquitinated protein (FANCD2), a helicase (FANCJ/BACH1/BRIP1) and a breast/ovarian cancer susceptibility protein (FANCD1/BRCA2), appear to cooperate in a pathway leading to the recognition and repair of damaged DNA. Molecular interactions among FA proteins and responsible proteins for other chromosome instability syndromes (BLM, NBS1, MRE11, ATM, and ATR) have also been found. Furthermore, inactivation of FA genes has been observed in a wide variety of human cancers in the general population. These findings have broad implications for predicting the sensitivity and resistance of tumors to widely-used anti-cancer DNA crosslinking agents (cisplatin, mitomycin C, and melphalan). Here, we summarize recent progress in the molecular biology of FA and discuss roles of the FA proteins in DNA repair and cancer biology.
**Introduction**

Many hematologists are familiar with Fanconi Anemia (FA) as a cause of childhood-onset aplastic anemia. However, this rare genetic disease now attracts broader attention from cancer biologists and basic researchers. FA is a rare chromosome instability syndrome characterized by aplastic anemia, cancer/leukemia susceptibility, and cellular hypersensitivity to interstrand DNA crosslinking agents, such as cisplatin, mitomycin C (MMC), diepoxybutane (DEB), and melphalan. Treatment with these agents causes increased chromosome breakage in cells derived from patients with FA. Because of this hypersensitivity to DNA crosslinking agents, many researchers have speculated that the primary defect of FA cells is in DNA damage response or DNA repair, although until recently direct evidence of connection between FA and DNA repair had not been shown.

Recent identification of the responsible genes for FA has changed our view of the molecular pathogenesis of the disease. FA can be divided into at least twelve complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, and M) defined by cell fusion studies, and eleven of the twelve responsible FA genes have been identified (Table 1)(Figure 1). Proteins encoded by FA genes (FA proteins) include a ubiquitin ligase (FANCL/PHF9/POG), a monoubiquitinated protein (FANCD2), a helicase (FANCJ/BACH1/BRIP1), and a protein with helicase motifs and a nuclease motif (FANCM) and a well-known breast/ovarian cancer susceptibility protein (FANCD1/BRCA2). FA proteins (including BRCA2) and another well-known breast/ovarian cancer susceptibility protein, BRCA1, cooperate in a common DNA repair process which is required for cellular resistance to DNA interstrand crosslinks (ICL), and a concept of the "Fanconi Anemia-BRCA pathway" (or FA-BRCA network) has been proposed.

Furthermore, molecular and functional interaction of FA proteins with proteins (ATM, MRE11, BLM, NBS1, and ATR) responsible for other rare genetic chromosome instability syndromes (ataxia telangiectasia (AT), AT-like disorder (ATLD), Bloom syndrome, and Seckel syndrome, respectively) in DNA damage response is now evident. Therefore, the FA pathway appears to regulate DNA repair.

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**Table 1.** Twelve complementation groups and responsible genes for Fanconi Anemia

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Responsible gene</th>
<th>FA patients, estimated, %*</th>
<th>Chromosome location</th>
<th>Protein kDa</th>
<th>Requirement for FANCD2 monoubiquitination</th>
<th>Main function of the protein</th>
<th>Comments</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>FANCA</td>
<td>57</td>
<td>15q24.3</td>
<td>163</td>
<td>+</td>
<td>FA core complex</td>
<td></td>
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<td>B</td>
<td>FANCB/FANP53</td>
<td>0.3</td>
<td>16p22-23.1</td>
<td>96</td>
<td>+</td>
<td>FA core complex</td>
<td>cytoplasmic functions</td>
</tr>
<tr>
<td>C</td>
<td>FANCC</td>
<td>15</td>
<td>5q22.3</td>
<td>63</td>
<td>+</td>
<td>FA core complex</td>
<td>Rad51 recruitment</td>
</tr>
<tr>
<td>D1</td>
<td>FANCD1/BRCA2</td>
<td>4</td>
<td>13q12-13</td>
<td>380</td>
<td>-</td>
<td>Rad51 recruitment</td>
<td>monoubiquitinated protein</td>
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<tr>
<td>D2</td>
<td>FANCD2</td>
<td>3</td>
<td>9p22.3</td>
<td>155,162</td>
<td>+</td>
<td>FA core complex</td>
<td>direct binding to FANCL2</td>
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<td>E</td>
<td>FANLE</td>
<td>1</td>
<td>6p21-22</td>
<td>90</td>
<td>+</td>
<td>FA core complex</td>
<td>FA core complex</td>
</tr>
<tr>
<td>F</td>
<td>FANCF</td>
<td>2</td>
<td>11p15</td>
<td>42</td>
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<td>G</td>
<td>FANCG/XRCC5</td>
<td>9</td>
<td>4q11</td>
<td>48</td>
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<td>FA core complex</td>
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<tr>
<td>I</td>
<td>not identified</td>
<td>--</td>
<td></td>
<td>?</td>
<td>+</td>
<td>FA core complex</td>
<td>S -&gt; S DNA helicase/ATPase</td>
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<tr>
<td>J</td>
<td>FANCL/BACH1/BRIP1</td>
<td>1.6</td>
<td>17q22-q24</td>
<td>130</td>
<td>--</td>
<td>binding to BRCA1</td>
<td>helicase/nuclease motif</td>
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<tr>
<td>L</td>
<td>FANLL/FANM/FANL1</td>
<td>0.1</td>
<td>4p16.1</td>
<td>43</td>
<td>+</td>
<td>FA core complex, ubiquitin ligase</td>
<td></td>
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<tr>
<td>M</td>
<td>FANCS/FANCC/POG</td>
<td>rare</td>
<td>14q21-23</td>
<td>250</td>
<td>+</td>
<td>FA core complex, DNA helicase/ATPase</td>
<td>helicase/nuclease motif</td>
</tr>
</tbody>
</table>

*unpublished data from the International Fanconi Anemia Registry (IFAR) (kindly provided by Dr. Arleen D. Auerbach)
Ubiquitination is a posttranslational modification in which a 76-residue small protein, ubiquitin, is covalently attached to a target protein (reviewed in reference 28). Polyubiquitination is well known for its function in targeting proteins for degradation by the 26 S proteasome. In contrast, monoubiquitination (conjugation of one ubiquitin molecule onto a protein) is a reversible signal regulating either protein targeting, membrane trafficking, histone function, transcription regulation, or DNA repair 29. Monoubiquitination of one of the FA proteins, FANCD2, is a critical step in the activation of the FA-BRCA pathway, and most of the other FA proteins are required for this monoubiquitination event 11.

Importantly, defects of the FA genes have been found in a wide variety of human cancers in the general population (non-FA patients) 30-40. Defects of DNA repair and cell cycle checkpoints, such as the defects of the FA pathway, are possible mechanisms of genomic...
instability in cancer and may also be responsible for the hypersensitivity of cancer cells to certain types of chemotherapeutic drugs and radiation. Disruption of FA genes may turn out to be a useful predictor of sensitivity to chemotherapy with widely-used anti-cancer DNA crosslinking agents (cisplatin, MMC, and melphalan) \cite{30,38,41}.

Thus, understanding the molecular biology of FA is important, not only for hematologists who manage patients with FA, but also for cancer biologists interested in the mechanism of drug sensitivity/resistance and for basic scientists working on DNA repair and/or ubiquitin biology.

Clinical course of FA

FA is a rare autosomal (all complementation groups except for FA-B group) or X-linked (FA-B group) recessive disease, clinically characterized by multiple congenital abnormalities, bone marrow (BM) failure, and cancer susceptibility. The clinical course of FA has been extensively reviewed \cite{2,3,42}. The prevalence of FA is estimated to be 1-5 per million and heterozygous carrier frequency is estimated to be 1 in 300, although the true frequency is probably higher \cite{1,2}.

FA patients show extreme clinical heterogeneity \cite{2,3}. The median age at diagnosis is 6.5 years for male patients, and 8 years for female patients, although the age at diagnosis ranges from 0 to 48 years. The male to female ratio is 1.24. The median survival age has improved to 30 years in patients reported between 1991 and 2000 \cite{3}. Survival was 19 years in those reported between 1981-1990\cite{3}. The common congenital defects seen in FA patients includes short stature (51%), abnormalities of the skin (55%), upper extremities (43%), head (26%), eyes (23%), kidneys (21%), ears (11%) and developmental disability (11)\cite{3}. Thirty two percent of male FA patients show abnormal gonads, although abnormal gonads has been described in only 3% of female FA patients \cite{3}. A significant percentage (25%-40%) of the FA patients were reported to be physically normal \cite{3}.

The hematologic complications of FA have also been extensively reviewed \cite{2,3}. FA patients develop BM failure, typically during the first decade of life. The actuarial risk of developing BM failure is 90% by 40 years of age \cite{43}. At least 20% of patients with FA also develop malignancies \cite{43}. The actuarial risks of developing hematologic and nonhematologic neoplasms are 33% and 28%, respectively, by 40 years of age \cite{43}. The median age of patients who develop cancer is 14 years for acute myelogenous leukemia (AML), 13 years for liver tumors, and 26 years for all solid tumors \cite{44}. In approximately 25% of FA patients with cancer, the malignancy preceded the diagnosis of FA \cite{44}. Some FA patients develop multiple cancers \cite{44}.

The most common malignancies in FA patients are AML and myelodysplastic syndrome. The most frequently reported chromosomal abnormalities in FA-associated leukemias are monosomy 7 and duplication of 1q \cite{45}. A recent study identified a high incidence of chromosome 3q abnormalities in BM cells of FA patients. Gain of 3q is strongly associated with a poor prognosis \cite{46}.

FA patients are also susceptible to solid tumors, such as head and neck squamous cell carcinoma (HNSCC), gynecological squamous cell carcinoma (SCC), esophageal carcinoma, liver tumors, brain tumors, skin tumors and renal tumors \cite{43,44,47}. Patients receiving androgen therapy for BM failure are prone to liver tumors, suggesting that androgens promote carcinogenesis in these patients \cite{44}. A high incidence (84%) of human papilloma virus (HPV) DNA and lack of p53 mutation in SCC (anogenital, or head and neck) in FA patients was
reported, but another group reported conflicting result (lack of HPV positivity and high p53 mutation prevalence). The role of HPV should be further investigated, since HPV infection may be prevented by prophylactic HPV vaccination.

There is no clear evidence of increased cancer susceptibility in heterozygous carriers of mutation in FA genes, except for BRCA2, whose heterozygous carriers have increased susceptibility to breast/ovarian and other cancers. FA gene (FANCA, FANCC, FANCD2, FANCE, FANCF and FANCG) mutations are rare in BRCA1/2 mutation-negative families with inherited breast cancer. Two missense germline mutations of BACH1/FANCJ associated with early-onset breast cancer were reported, but germline mutations in FANCJ are rare in breast and ovarian cancer families.

**Diagnosis and treatment of FA**

Treatment of FA cells with DNA crosslinking agents, such as DEB, causes increased chromosome breakage and marked G2 accumulation. The DEB-induced chromosome breakage assay (DEB test) is widely used as a standard diagnostic test for FA. FA subtyping (determination of the complementation group of each FA patient) has become increasingly important. Some patients with other rare chromosome breakage syndrome, such as NBS, can have a positive DEB test. Furthermore, a relationship between genotype and phenotype has been reported. Patients in complementation group C (FA-C) had a significantly poorer survival than patients in groups A and G. FA-D1 subtype is associated with increased predisposition to medulloblastoma, Wilms tumor, and acute leukemia in early childhood, and is clinically different from other subtypes. Family members of FA-D1 patients may be carriers of mutation in BRCA2, and may be predisposed to cancers. Therefore, in order to confirm the diagnosis, to distinguish FA from other chromosome breakage disorders, and to manage each FA patient and family better, FA subtyping should be performed routinely in the near future. For subtyping, cell fusion assays have been used, but a combination of retroviral gene transfer and G2 accumulation assay or a combination of retroviral gene transfer and FANCD2 immunoblotting are also useful.

The treatment of FA has been extensively reviewed elsewhere. For BM failure, androgens and hematopoietic growth factors are effective in many cases, but most FA patients become refractory to these treatments. For such patients, hematopoietic stem cell transplantation (SCT) is performed, providing a donor is available. The outcome of SCT has improved. Fludarabine-based regimens have proven to be a significant advance. Now that many FA patients survive their hematopoietic problems, prevention, surveillance and treatment of solid tumors are becoming more and more important. Since radiotherapy/chemotherapy may cause severe side effects, surgery is the primary therapy for solid tumors in FA. As a new modality of therapy, gene therapy will be an option in the future.

**Complementation groups and FA genes/proteins.**

The twelve FA complementation groups and the responsible genes/proteins are summarized in Table 1 and Figure 1. Eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM) form a nuclear protein complex (the FA core complex), which is required for monoubiquitination of another FA protein, FANCD2 (Figure 2A). Monoubiquitination of FANCD2 is required for translocation of FANCD2 to
chromatin and for nuclear foci formation of FANCD2, which may reflect accumulation of FANCD2 protein at the sites of DNA damage and repair.11,61

Figure 2. Current model of the Fanconi Anemia pathway. (A) The FA proteins are depicted in the normal cell nucleus. In response to DNA damage, or during normal S phase progression, the FANCD2 protein is monoubiquitinated on lysine 561. Efficient monoubiquitination requires several proteins including ATR, RPA, and an intact FA core complex. Monoubiquitination of FANCD2 targets the protein into nuclear foci and chromatin fraction where it interacts with BRCA2. The function of monoubiquitinated FANCD2 (FANCD2-Ub) in chromatin remains unknown, and it may regulate HR repair and/or translesion DNA synthesis. USP1 negatively regulates monoubiquitination of FANCD2. (B) This figure depicts several chromatin proteins that appear to interact in the repair of the DNA interstrand crosslink (See text).
FANCA contains two overlapping bipartite nuclear localization signals (NLS), five functional leucine-rich nuclear export sequences (NESs) and a partial leucine zipper sequence. The nuclear export of FANCA is regulated in a CRM1-dependent manner.

FANCB (FAAP95) contains a putative bipartite NLS. FANCB gene is located on the X chromosome, and all of the reported FA-B patients are male.

FANCC is a component of the nuclear FA core complex, but localizes both in nucleus and cytoplasm. Some functions of FANCC outside of the FA core complex, such as involvement in Jak/STAT signaling and apoptotic signaling, have been also proposed.

The gene for FA-D1 subtype, FANCD1, is identical to a breast/ovarian cancer susceptibility gene, BRCA2. Carriers of mono-allelic germline mutation of BRCA2 have breast/ovarian cancer susceptibility, but patients with biallelic germline mutations develop FA-D1 subtype of FA. BRCA2 is not a component of the FA core complex, nor is it required for monoubiquitination or nuclear foci formation of FANCD2. A principal function of BRCA2 is regulation of homologous recombination (HR) repair through control of RAD51 recombinase (eukaryotic homologue of bacterial RecA). BRCA2 also has other functions, such as stabilization of stalled replication forks, regulation of cytokinesis, etc. BRCA2 protein has eight copies of 30-40 amino acid motif (BRC repeats). The BRC repeats directly bind to RAD51 protein, which can form nucleoprotein filament with single-stranded DNA (ssDNA). The C-terminal region of BRCA2 is also implicated in RAD51 binding. The C-terminal domain (CTD) has five domains; a helical domain (HD), three oligonucleotide/oligosaccharide-binding folds (OB1, OB2, and OB3) and a tower domain (TD) inserted in OB2. The OB folds bind to ssDNA. The TD interacts with double stranded DNA (dsDNA). BRCA2 may bind to ssDNA-dsDNA junction of resected DNA ends of double strand DNA breaks and help RAD51 loading on ssDNA. DSS1 (deleted in split-hand/split foot syndrome) interacts with BRCA2 through the HD, OB1, and OB2 domains. DSS1 is required for stability of BRCA2.

FANCD2 does not have any known functional motifs. Normal cells express two isoforms of the FANCD2 protein, a non-ubiquitinated form (155kDa) and a monoubiquitinated form (162kDa). Monoubiquitination occurs on a single residue, lysine 561. Monoubiquitinated FANCD2 protein is mainly located in chromatin fraction. The C-terminal region of FANCD2 contains several acidic amino acid residues, and is required for the function of FANCD2 in ICL resistance, although this region is not required for monoubiquitination nor chromatin localization of FANCD2.

FANCE is a nuclear protein required for nuclear accumulation of FANCC. FANCE interacts with monoubiquitinated FANCD2 and BRCA2 in the chromatin fraction. FANC has a region of homology with the prokaryotic RNA-binding protein ROM, but this homology appears to be non-significant. FANC acts as a flexible adaptor protein required for the assembly of the FA core complex.

FANCG/XRCC9 was originally cloned as a cDNA that partially corrects the MMC sensitivity of a Chinese hamster mutant UV40 cell line. FANCG has seven tetratricopeptide repeat motifs (TPRs). TPRs are degenerate 34-amino acid repeat motifs mediating protein-protein interactions. Serines 383 and 387 on FANCG are phosphorylated in M phase, presumably by cdc2. These two sites are important for exclusion of FANCG from chromatin in mitosis. Phosphorylation of serine 7 of FANCG is upregulated after MMC treatment.
FA-I cells lack monoubiquitination of FANCD2 but form the FA core complex normally. The still-unidentified FANCI protein is considered to be a factor required for monoubiquitination of FANCD2, but not a subunit of the FA complex. FANCI/BACH1/BRIP1 is a DNA-dependent ATPase and a 5'-to-3' DNA helicase (DEAH helicase) that directly binds to the BRCT domain of BRCA1. BRCT domain is a phosphoprotein-binding domain, and phosphorylation of serine 990 of BACH1 is required for BRCA1-BACH1 interaction. FANCJ contains the seven helicase-specific motifs, and C-terminal extension which has 39% homology with synaptonemal complex protein1, a major component of the transverse filaments of developing meiotic chromosomes. FANCJ is not required for monoubiquitination of FANCD2.

FANCL/PHF9/POG (FAAP43) has three WD40 repeats and a PHD finger motif. WD40 repeats are known to mediate protein-protein interactions. The PHD motif is a variant of RING finger, and sometimes shows ubiquitin ligase activity. FANCL has an autoubiquitin ligase activity in vitro, is required for FANCD2 monoubiquitination in vivo, and is presumed to be the catalytic subunit of the FA core complex as an ubiquitin ligase for FANCD2. However, in vitro reconstitution of monoubiquitination of FANCD2 by FANCL has not been demonstrated. The mouse homolog of FANCL was identified as a gene responsible for the phenotype of gcd (germ cell-deficient) mice, which showed reduced fertility with defective proliferation of germ cells.

FANCM (Hef)(FAAP250) protein is a component of the FA core complex, and contains the seven helicase-specific motifs, and one degenerate endonuclease domain. FANCM has ssDNA and dsDNA-stimulated ATPase activity and DNA translocase activity. So far, the helicase activity or endonuclease activity of FANCM has not been shown. FANCM is closely related to the archaean protein Hef (helicase-associated endonuclease for fork-structured DNA), which has a functional helicase domain and a functional endonuclease domain and resolves stalled replication forks. Human XPF/ERCC4 endonuclease (the responsible protein for xeroderma pigmentosum complementation group F) and yeast MPH1 helicase also have high homology to archaean Hef. These proteins may have derived from a common ancestor and may have diverged. FANCM is phosphorylated in response to DNA damage.

Interaction of the FA proteins: The FA pathway.

Because cells from all subtypes of FA are hypersensitive to ICL, the FA proteins have been considered to cooperate in a common DNA damage response or DNA repair pathway (the FA pathway) (Figure 2A). The pathway is also referred to as "the FA-BRCA pathway" or "the FA-BRCA network". The critical steps in the pathway are the monoubiquitination and nuclear foci formation of the FANCD2 protein.

Eight FA proteins (A, B, C, E, F, G, L and M) and one unidentified protein (FAAP100) form a nuclear complex (FA core complex). Because monoubiquitination of FANCD2 is completely dependent on the FA core complex and FANCL has ubiquitin ligase activity, the FA core complex is presumed to be a multi-subunit ubiquitin ligase complex responsible for monoubiquitination of FANCD2. FA core complex, BLM, RPA and topoisomerase III alpha form a larger complex called BRAFT (for BLM, RPA, FA, and Topo III alpha). BLM is not required for monoubiquitination of FANCD2.

Monoubiquitination of FANCD2 is required for nuclear foci formation, chromatin localization, and function of FANCD2 to restore resistance to ICL in FA-D2 cells. In response to DNA damage, the FA pathway becomes activated. After treatment with DNA
damaging agents, such as ionizing radiation (IR), ultraviolet, DNA crosslinking agents and hydroxyurea, FANCD2 is monoubiquitinated and targeted to BRCA1/BRCA2/RAD51-containing nuclear foci, presumably at the sites of DNA damage and repair \(^{11,80,92}\). FANCD2 also colocalizes with a DNA damage-activated signaling kinase, ATR, and RPA in DNA-damage induced nuclear foci \(^{22}\), and partially colocalized with FANCE \(^{79}\), FANCC \(^{93}\), NBS1 \(^{17}\), BLM \(^{19,20}\), and FANCJ \(^{12}\). FANCG is reported to colocalize with BRCA2 and RAD51 \(^{94}\), suggesting colocalization of FANCG and FANCD2. All of these factors are required for cellular resistance to ICL.

ATR is required for the DNA damage-induced monoubiquitination and nuclear foci formation of FANCD2, although its specific mechanism in the process remains unclear \(^{22}\). FANCD2 itself can be directly phosphorylated by ATR \textit{in vitro} \(^{22}\), and phosphorylation of FANCD2 in response to ICL is reported to be ATR dependent \(^{23}\). Therefore, ATR-mediated phosphorylation of FANCD2 may enhance monoubiquitination of FANCD2. On the other hand, ATR may phosphorylate some components of FA core complex and may enhance ubiquitin ligase activity of the complex. The recent finding of DNA damage-inducible FANCM phosphorylation is consistent with the latter hypothesis \(^{5}\).

BRCA1 is a RING finger protein with ubiquitin ligase activity. BRCA1 is required for efficient DNA damage-inducible increase of monoubiquitinated FANCD2 in a breast cancer cell line, HCC1937 \(^{11}\), but this phenomenon is not reproducible in HeLa cells exposed to BRCA1 siRNA, nor in BRCA1 deleted chicken DT40 cells \(^{95}\). Therefore, the \textit{in vivo} role of BRCA1 for monoubiquitination of FANCD2 is questionable, although BRCA1 together with BARD1 can monoubiquitinated FANCD2 \textit{in vitro} \(^{95}\). BRCA1 is required for DNA damage-inducible nuclear foci formation of FANCD2 in any of these cells \(^{11,95}\). BRCA1 also facilitates FANCJ \(^{52}\) and RAD51 nuclear foci formation after DNA damage.

In the absence of DNA damage, FANCD2 is monoubiquitinated and forms nuclear foci with BRCA1 and RAD51 in S phase of the cell cycle \(^{96}\), and for this activation in S phase, ATR is not required \(^{22}\). The functional significance and mechanism of activation of the pathway in S phase remains unknown.

Monoubiquitinated FANCD2 is preferentially retained in the chromatin fraction \(^{61,80}\). In chromatin fraction, monoubiquitinated FANCD2 can be co-immunoprecipitated with BRCA2 and FANCE \(^{80}\). Monoubiquitinated FANCD2 is required for the increase of nuclear foci of BRCA2 and RAD51 in response to DNA damage \(^{80}\), suggesting a role of BRCA2 and RAD51 downstream of monoubiquitinated FANCD2. RAD51 foci formation is an important marker for the integrity of upstream HR machinery. BRCA2 is required for the increase of RAD51 foci after DNA damage. Whether FA proteins other than BRCA2 also increase the assembly of RAD51 foci is controversial. Some reports claim that FA core complex proteins are required for efficient RAD51 foci formation in response to certain types of DNA damage \(^{21,80,97}\). Other reports claim that these FA proteins are not required for DNA-damage inducible RAD51 foci formation \(^{12,98-101}\). This discrepancy may reflect the use of different anti-RAD51 antisera and different degrees of genotoxic stress.

A deubiquitinating enzyme, USP1, is a negative regulator of the FA pathway \(^{102}\). Knockdown of USP1 causes increased monoubiquitination of FANCD2, and cellular resistance to MMC. Although direct deubiquitination of FANCD2 by USP1 has not been demonstrated, USP1 is likely to be the deubiquitinating enzyme for monoubiquitinated FANCD2 \textit{in vivo}.

Monoubiquitination of PCNA is mediated by RAD 6 and RAD18 and is implicated in DNA polymerase switch from a replicative polymerase to a translesion synthesis (TLS)
polymerase, DNA polymerase eta (Rad30, XPV), at the site of blocked replication forks. These factors are part of post-replication repair (PRR) pathway, and are also required for cellular resistance to ICL. Surprisingly, USP1 deubiquitinates monoubiquitinated PCNA (Huang TT and D’Andrea AD, unpublished data), suggesting a common shut-off mechanism of two different DNA repair pathways required for cellular resistance to ICL, the FA pathway and the PRR pathway.

Other proteins interacting with FA proteins include alpha Spectrin II, FAZF, SNX5, NADPH cytochrome P450 reductase, a molecular chaperone GRP94, cdc2, GSTP1, CYP2E1, BRG1, STAT1, Hsp70, PKR, menin, and many other proteins identified in yeast two hybrid screens (summarized in reference 105). The functional relevance of these protein-protein interactions remains largely unknown.

**Localization of FA proteins in chromatin**

Until recently, the FA core complex was believed to be a soluble nuclear complex whose primary function was to monoubiquitinate FANCD2. The identification of two new FA proteins with helicase motifs (FANCM and FANCJ) provide strong evidence that the FA complex proteins may in fact move in and out of the chromatin, depending on the state of DNA damage or cell cycle phase.

The FA core complex does not simply function as the ubiquitin ligase for FANCD2. Increasing evidence suggests roles of components of FA core complex in the chromatin. First, FANCA, FANCC and FANCG proteins are localized to chromatin in G1/S/G2 phases and chromatin localization of these proteins increases after MMC treatment. Second, FANCE and FANCC are reported to form nuclear foci and colocalize with FANCD2 after DNA damage. FANCG also forms ICL-induced nuclear foci and colocalizes with BRCA2 and RAD51. Third, FANCE interacts with BRCA2 and FANCD2 in chromatin. Fourth, FANCM has a helicase motif and a degenerate nuclease motif, suggesting an important role for the FA core complex in moving along the DNA and sensing damage. FANCM also has DNA-stimulated ATPase activity and translocase activity, and it obviously interacts with DNA. Consistent with these findings, a recent report using a chicken FANCD2-monoubiquitin fusion protein suggests at least two more functions of the FA core complex other than monoubiquitination of FANCD2. According to this report, the monoubiquitination of FANCD2 is not sufficient for translocation of FANCD2 into chromatin. Even after FANCD2 is monoubiquitinated, the FA core complex is required for translocation of FANCD2 into chromatin. Furthermore, even after FANCD2 is localized in chromatin, the FA core complex is required for cellular resistance to ICL. These findings indicate that FA core complex is not merely the ubiquitin ligase for FANCD2. The FA core complex may ubiquitinate other substrates, or may have functions other than ubiquitination.

Other FA proteins are also known to interact with DNA. FANCJ is a DNA-dependent ATPase and a 5'-to-3' DNA helicase, which clearly interacts with DNA. Monoubiquitinated FANCD2 is mainly localized in chromatin fraction and purified FANCD2 protein has direct DNA binding activity with specificity for dsDNA ends and Holliday junctions, suggesting a direct role of FANCD2 in DNA repair. BRCA2 interact with DNA through OB folds and tower domain, and recruits a recombinase, RAD51.

Some non-FA proteins interacting with FA proteins also interact with DNA. BLM is a part of BRAFT complex, and ICL-induced nuclear foci formation of BLM is dependent on
FANCC \(^{19,20}\), FANCG \(^{19}\), and FANCD2 \(^{20}\). ICL-induced phosphorylation of BLM is dependent on FANCC and FANCG, but not on FANCD2 \(^{19}\). BLM colocalizes with FANCD2 in ICL-induced nuclear foci, and can be co-immunoprecipitated with monoubiquitinated FANCD2 \(^{19,20}\). BLM-deficient cells are sensitive to ICL to some extent \(^{19}\). BLM has 3’ to 5’ DNA helicase activity and is associated with topoisomerase III alpha, which can break and rejoin DNA to alter its topology, and ssDNA binding protein, RPA.

The NBS1-MRE11-RAD50 complex also interacts with FA proteins \(^{17,21}\). MRE11 has 3’-5’ dsDNA exonuclease, and ssDNA and dsDNA endonuclease activity \(^{25}\). NBS1 deficient cells and MRE11 deficient cells are sensitive to ICL \(^{17}\). FANCD2 and NBS1 partially colocalize in MMC-induced nuclear foci and are co-immunoprecipitated \(^{17}\). FA core complex is required for phosphorylation of NBS1 and nuclear foci formation of MRE11 in response to ICL specifically \(^{21,110}\).

XPF is an endonuclease required for incision of ICL and forms heterodimer with ERCC1. XPF deficient cells and ERCC1 deficient cells are hypersensitive to ICL. XPF colocalizes with FANCA in ICL-induced nuclear foci \(^{111}\). A related endonuclease, MUS81-Eme1, which is required for cellular resistance to ICL, may also have interactions with the FA pathway.

TLS DNA polymerases (REV1 and REV3) are also implicated in the FA pathway. Chicken DT40 cells deficient in REV3 or REV1 show crosslinker hypersensitivities that are epistatic to FANCC-deficient cells \(^{112}\). Furthermore, REV1 and FANCD2 colocalize in nuclear foci upon replication arrest \(^{112}\). This raises the interesting possibility that FA proteins and error prone TLS polymerases work in a common pathway, which is consistent with the reported hypomutability in FA cells \(^{113}\).

The important question is how these factors (helicases, nucleases, a topoisomerase, DNA polymerases, a recombinase and other DNA binding proteins) assemble in chromatin and repair damaged DNA lesions (especially ICL) (Figure 2B). Step-by-step analysis of DNA repair intermediates from ICL lesions will be important. Investigation of the requirement of each factor for the formation of such intermediates will be required in order to envision the whole repair process \(^{114}\).

**Cellular Defects of DNA repair in FA**

Increasing evidence suggests that FA cells are defective in HR, although there is some inconsistency among reports (Table 2). BRCA2 deficient cells show a severe decrease in overall HR efficiency \(^{69,115,116}\). A mild HR defect in other FA gene deficient cells (FA-A \(^{116,117}\), C \(^{20,112}\), D2 \(^{99,116}\), G \(^{100,116}\) and J \(^{12}\)) have been reported, although normal HR efficiency in FA-C \(^{89}\), D2 \(^{101}\), J \(^{89}\) and M \(^{118}\) cells have also been reported. Some of these discrepancies may be explained by difference in cell types used and systems used for measurement of HR. Overall, there seems to be at least a mild defect of HR in FA cells.

Spontaneous sister chromatid exchange (SCE) is elevated in FANCC \(^{20,112}\), FANCD2 \(^{99}\), or FANCI \(^{89}\) deficient chicken DT40 cells. These are intriguing data, because 1) SCE is thought to be mediated by HR, and 2) in human FA cells, spontaneous SCE is not elevated. One speculative explanation is that in FA cells, a sub-pathway of HR which is not required for spontaneous SCE may be affected \(^{99}\), and that something which masks increased SCE in human FA cells may be missing in chicken DT40 cells. Interestingly, in DT40 cells, increased SCE in FANCC deficient cells is epistatic with BLM deficient cells, indicating a functional linkage between FANCC and BLM in suppressing SCE \(^{99}\).
BRCA2 deficient cells show increased single strand annealing (SSA)\textsuperscript{115,116}. In contrast, BRCA1, FANCA, FANCG or FANCD2 deficient cells show reduced SSA\textsuperscript{115-117}, suggesting a role of FA proteins (other than BRCA2) in common steps for both SSA and HR.

HR activity is elevated in FA-C, and FA-G cells in assays using extra-chromosomal plasmid-based templates\textsuperscript{119}, suggesting that regulation of HR by FA proteins outside of chromosome may be different from regulation in chromosome. In plasmid-based end-joining assays, the end-joining fidelity of DNA double strand breaks is low in FA cells\textsuperscript{120}.

FA cells are generally not hypersensitive to ionizing radiation (IR)\textsuperscript{121}, but clinically some FA patients show increased sensitivity to IR (unexpected toxicity due to radiation therapy)\textsuperscript{122}. In the absence of DNA-PK, which is an essential component of non-homologous end joining (NHEJ) repair and which phosphorylates many substrates in response to DNA damage\textsuperscript{123}, \textit{Fancd2} knockout sensitizes fibroblasts and mice to IR\textsuperscript{124}. These results suggest that normal NHEJ repair may mask the IR-hypersensitivity of FA cells. This may explain inconsistent IR sensitivities among FA cells and patients.

Common fragile sites are chromosomal loci that preferentially exhibit gaps and breaks when cells have been cultured under replicative stress. FA cells, ATR-deficient cells and BRCA1-deficient cells show increased common fragile site instability, further supporting the idea of FA pathway interactions\textsuperscript{125}.

**Function of FA proteins in intra S phase cell cycle checkpoints**

The \textit{FANCD2} protein has been implicated in IR-induced intra S phase checkpoint\textsuperscript{16}. After IR exposure, \textit{FANCD2} is phosphorylated by an IR-activated signaling kinase called ATM on several residues including serine 222. ATM kinase phosphorylates and activates proteins involved in cell cycle checkpoint responses, including p53, CHK2, NBS1, SMC1, BRCA1 and \textit{FANCD2}\textsuperscript{24}. Normal cells stop synthesizing DNA in response to exposure to IR; this response is
called intra S phase checkpoint. Cells deficient in ATM, or some substrates of ATM, such as CHK2, NBS1, SMC1, BRCA1, and FANCD2, do not stop synthesizing DNA after exposure to IR. IR-inducible ATM-dependent phosphorylation of FANCD2 on serine 222 is required for establishment of IR-induced intra-S phase checkpoint, but is not required for ICL resistance. This phosphorylation is also dependent on NBS1. In contrast, monoubiquitination of FANCD2 on lysine 561 is required for ICL resistance, but not required for intra-S phase checkpoint. Therefore, FANCD2 has two independent functions, resulting from two independent post-translational modifications.

FA cells fail to arrest DNA synthesis in response to ICL whereas normal cells arrest DNA replication. This means FA cells have a defect in ICL-induced S phase checkpoint function, which is similar to a defect in IR-induced intra S phase checkpoint function seen in ATM, NBS1, or FANCD2 deficient cells. The ICL-induced ATR-dependent phosphorylations of NBS1 and FANCD2 seem to be required for the establishment of ICL-induced intra S phase checkpoint.

Other functions of FA proteins

FA cells are hypersensitive to oxygen, and FA proteins have been implicated in the handling of oxidative stress. Interaction of FANCC protein and some cytoplasmic proteins involved in handling of reactive oxygen species (for example, GSTP1) has been reported. FANCC has also been implicated in JAK/STAT signaling and apoptotic signaling. These functions of FA proteins, which appear to fall outside of DNA repair, are reviewed elsewhere and are beyond the scope of this article.

Cancer in murine models for FA

Generation of Fanca, Fancc, Fancg, Fancd2, Fanca-Fancc double, and Fancl/Pog knockout mice have been reported. Importantly, some of these FA mice develop tumors. Fancd2 knockout mice develop tumors including adenocarcinoma and lymphoma. Fanca knockout mice develop lymphoma, sarcoma, and ovarian granulosa cell tumor. Fancg knockout mice develop mammary adenocarcinoma and histiocytic sarcoma. Fance knockout reduces latency to tumor development of p53-/- and p53+/- mice. Heterozygosity for p53 (p53+/-) accelerates epithelial tumor formation in Fancd2 knockout mice. Many Brca1 or Brca2 deficient mice models with cancer susceptibility have been published (reviewed in reference). Those results demonstrate the importance of the FA pathway in tumor suppression.

Inactivation of the FA pathway in human cancer in the general population

Abnormalities of the FA pathway have been identified in a wide variety of human cancers, suggesting a molecular mechanism for their sensitivity to chemotherapy with DNA crosslinking agents (cisplatin, MMC, and melphalan). Table 3 summarizes recent reports describing abnormalities of the FA genes (except for BRCA2) in cancer in the general population. Abnormalities of BRCA1/2 in cancer have been reviewed elsewhere. Ovarian tumor cells are initially sensitive to cisplatin, but become refractory to cisplatin over time. Understanding the molecular basis of cisplatin sensitivity and acquired resistance is critical to the management of this tumor. In two ovarian cancer cell lines (TOV-21G and 2008),
methylation of the promoter region of the \textit{FANCF} gene causes suppression of FANCF expression, and increased sensitivity to MMC and cisplatin \cite{140}. \textit{FANCF} was partially demethylated, and FANCF expression was restored in a cisplatin-resistant derivative of 2008 cell line, suggesting that reactivation of the FA pathway is a mechanism for acquired resistance. At least in the cell lines, the integrity of the FA pathway is one of the critical determinants of cisplatin resistance \cite{140}. In contrast, the significance of \textit{FANCF} methylation in clinical samples of ovarian cancer remains unclear. In the initial report, it was detected in 21\% of a relatively small number of samples \cite{30}, but one subsequent report with a larger number of samples failed to detect \textit{FANCF} methylation in stage III/IV epithelial ovarian tumors \cite{140}. The true prevalence in epithelial ovarian cancer may be lower than initially estimated. \textit{FANCF} methylation was found in 24\% of clinical samples of a relatively rare subtype of non-epithelial ovarian tumor, granulosa cell tumors \cite{35}.

For lung adenocarcinoma, \textit{FANCF} methylation is a significant predictor of poor survival, and for non small cell lung cancer, it is associated with a shorter duration of tobacco use \cite{33}. In lung SCC, increased \textit{p16INK4a} homozygous deletion occurred at higher frequency in those with \textit{FANCF} methylation \cite{145}. In HNSCC, \textit{FANCF} methylation is associated with a greater number of years of alcohol drinking \cite{33}. Interpretation of these associations is difficult, but \textit{FANCF} methylation seems to have some clinical significance in these types of cancers.

In cervical cancer, \textit{FANCF} methylation was frequently observed (30\%) \cite{34}. Patients younger than 45 years of age showed a higher frequency of \textit{FANCF} methylation, suggesting that \textit{FANCF} methylation may play a role in initiation or progression of cervical cancer in younger patients \cite{34}.

Germ cell testicular cancer (non-seminoma type) is usually highly responsive to cisplatin. \textit{FANCF} methylation was found in a small subset (6.7\%) \cite{36}, indicating that the role of \textit{FANCF} methylation in general cisplatin sensitivity of non-seminoma is limited.

\textit{FANCA} abnormalities are involved in a small subset of AML. \textit{FANCA} missense mutations were found in 7.6\% of adult AML, although functional relevance of most of these mutations is unknown \cite{142}. Heterozygous deletion of \textit{FANCA} with reduced expression of \textit{FANCA} was identified in 4\% of adult AML patients \cite{143}.

Inherited and somatic mutations of \textit{FANCC} and \textit{FANCG} are present in a subset of young-onset pancreatic cancer \cite{37}. Two germline truncating \textit{FANCC} mutations associated with loss of heterozygosity in tumor samples were identified in young onset pancreatic cancer cases \cite{39}, suggesting that inherited mutations in \textit{FANCC} may predispose to pancreatic cancer.

Melfalan is widely used in the treatment of multiple myeloma. A comparison of a melfalan-sensitive myeloma cell line and its melfalan-resistant derivative identified \textit{FANCF}...
as a gene expressed significantly higher in the resistant derivative. Modulation of FANCF expression in a myeloma cell line by siRNA or by overexpression of FANCF causes melphalan sensitivity or resistance, respectively. Whether the FA pathway is inactivated in clinical samples of myeloma remains to be investigated.

One possible therapeutic approach for DNA crosslinker-resistant cancers is sensitization of tumor to DNA crosslinkers using a specific small-molecule inhibitor of the FA pathway. As a proof-of-principle, it has been shown that suppression of the FA pathway by an adenovirus overexpressing dominant-negative FANCA sensitizes tumor cells to cisplatin.

Poly(ADP-ribose) polymerase (PARP) inhibitors selectively kill BRCA1/2 deficient tumor cells. Whether PARP inhibitors can selectively kill other FA protein-deficient tumor cells is an important question, given the wide variety of tumors with inactivation of the FA pathway.

Concluding comments and unanswered questions

The eleven identified FA proteins appear to cooperate in a common pathway, regulating the repair of interstrand DNA crosslinks. The characterization of the cloned FA genes and proteins has allowed new diagnostic approaches to FA and suggests novel treatment options for FA patients.

Critical questions remain for the FA pathway. First, although FANCD2 monoubiquitination is a required event in this pathway, little is known about the downstream function of this post-translational modification. For instance, monoubiquitinated FANCD2 may activate crosslink repair, bind to a novel TLS polymerase, and/or bind other DNA repair subunits. Second, the role of the pathway is primarily in DNA damage response. Yet, the role of the new DNA binding components (FANCM and FANCJ) in damage recognition versus DNA repair is largely unknown. Third, it will be important to identify the FANCI protein and perhaps other proteins in the pathway.

Finally, further examination of the role of the FA pathway in the cause and treatment of cancers in the general population is warranted. As is often the case with the study of a rare pediatric disease, FA research has already led to general insights to other more prevalent human diseases, such as aplastic anemia, infertility, and cancer.

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