

## CHAPTER 2

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# The Genetic Basis of Fanconi Anemia

Grover C. Bagby, Jr.\*

### Introduction

Seventy-five years ago, Dr. Guido Fanconi reported three siblings who exhibited both congenital defects and aplastic anemia.<sup>1</sup> Since then, we have learned that Fanconi anemia (FA) is a rare multigenic disorder (a prevalence of 1-5 per million<sup>2</sup>), that predisposes children and adults to life-threatening bone marrow failure, myelodysplasia,<sup>3</sup> acute nonlymphocytic leukemia (AML), and certain epithelial malignancies.<sup>4-7</sup> So far, the *sine qua non* of this disease is cytogenetic instability in vitro after exposure of FA cells to bifunctional alkylating agents.<sup>8,9</sup> Indeed, the current diagnostic test for Fanconi anemia, quantification of chromosomal breakage responses to alkylating agents, is based on this feature (reviewed in ref. 10). Classic clinical features such as growth retardation, small head size, *café-au-lait* spots, and radial ray defects can be strong diagnostic clues,<sup>11</sup> but FA can occur in patients without congenital defects and can be clinically ascertained in adulthood. In fact, some patients with very minimal blood count abnormalities have been identified only because they were siblings of known FA patients and were tested for that reason. Consequently, there is substantial phenotypic heterogeneity in FA and while some of it can be explained by genetic heterogeneity, certain of the clinical consequences are the result of gene-environment interactions.

### Genetic Heterogeneity

Somatic cell fusion studies have defined 11 complementation groups, FA-A, B, C, D1, D2, E, F, G, L, I and J.<sup>12</sup> All eleven can be accounted for by mutations of a gene unique to that group and nine of the genes have been cloned<sup>13-15</sup> (Table 1). Some of the proteins encoded by the normal FANC genes contain domains that suggest some functions (FANCG for example has a number of tetratricopeptide [TPR] repeats<sup>16</sup>). However, with the exception of FANCD1, now known to be identical to BRCA2,<sup>17</sup> the genes and proteins they encode have no strong homologies to one another or to other known proteins.

Two recurring themes have emerged from published studies on the function of the FA proteins; (1) that they function to protect against genotoxic stress at least in part by forming complexes with each other and facilitating monoubiquitination of FANCD2, and (2) that the proteins also interact functionally with other proteins governing survival-signaling pathways.<sup>18</sup> While this second function of FA proteins involves formation of complexes, they are probably complexes composed of molecules not found in the so called "core FA complex" that consists of FANCA, FANCB, FANCC, FANCE, FANCF, FANCG and FANCL. In fact, as will be mentioned in more detail below, the definition of the "core complex" is undergoing some substantial revision.

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\*Grover C. Bagby, Jr.—OHSU Cancer Institute, Departments of Medicine and Molecular and Medical Genetics, Oregon Health and Science University, Portland, Oregon 97239, U.S.A. Email: grover@ohsu.edu

**Table 1. The Fanconi anemia genes**

Gene	Prevalence of Mutations in Patients with FA (%)	Chromosomal Location	Exon Number	Amino Acid Residues (kDa)
FANCA	70	16q24.3	43	1455 (163)
FANCB	1	Xp22.31	10	859(95)
FANCC	10	9q22.3	14	558 (63)
FANCD1 (BRCA2*)	1	13q12.3	27	3418 (384)
FANCD2	1	3p25.3	44	1451 (155,162)
FANCE	5	6p21.3	10	536 (60)
FANCF	2	11p15	1	374 (40)
FANCG	10	9p13	14	622 (48)
FANCL (PHF9)	1	2p16.1	11	373 (43)
FANCI	Not known	Not known	Not known	Not known
FANCI	Not known	Not known	Not known	Not known

\*Although a BRCA2 null genotype is an embryonic lethal phenotype, certain homozygous BRCA2 mutations that lead to C-terminal truncations, lead to FA of the D1 complementation group.

## The *FANCA* Genes

Reviewed below are selected features of each *FANCA* gene and the proteins they encode. The chromosomal location and exon number of the genes and amino acid residues and molecular mass of the encoded proteins are listed in Table 1. A Fanconi Anemia Mutation Database, containing many FA mutations (but not all), is available ([www.rockefeller.edu/fanconi/mutate](http://www.rockefeller.edu/fanconi/mutate)). Currently, ascertainment of novel mutations is most often done by DHPLC analysis and sequencing of genomic DNA and cDNA. Apart from family studies, to confirm that an abnormality represents a mutation, complementation analysis (in which the wild type cDNA expressed in mutant cells corrects MMC/DEB sensitivity but the mutant cDNA does not) is most convincing. However, in light of the information now extant on key regions of FANCA genes, some sequence variations (especially large deletions) can be assigned as mutations by deduction.

### *FANCA*

Mutations of this gene are by far the most common in patients with FA, accounting for 65% of all cases. The spectrum and scope of discrete mutations of this gene are immense and include nonsense, missense, and splicing mutations as well as microdeletions, microinsertions, and duplications.<sup>19</sup> There may be more than 250 different mutant FANCA alleles, many of which are large intragenic deletions,<sup>20</sup> and only a few are common especially c.1115\_1118delTTGG (2% of FANCA alleles) and c.3788\_3790delTCT (5% of FANCA alleles).<sup>19</sup> The protein participates as a member of the core complex required for FANCD2 ubiquitination,<sup>21</sup> and binds to BRCA1,<sup>22</sup> but the functional role it plays in these complexes are unknown.

Post translational modifications of FANCA occur in response to environmental cues. For example, FANCA is redox-responsive and multimerizes with FANCG in response to oxidative stress.<sup>23</sup> Phosphorylation of FANCA also occurs in normal cells but not in most FA cells. In vitro phosphorylation studies identified a cytoplasmic serine kinase embedded in the core complex (sensitive to wortmannin) that phosphorylated FANCA on serines.<sup>24</sup> The functional consequences of this post-translational change and whether this putative FANCA kinase is the one that seems to be modulated by AKT<sup>25</sup> are unknown.

Like FANCC, FANCA also seems to participate directly in support of survival signal transduction pathways. For example, FANCA interacts with IKK2 and may thereby integrate with the I $\kappa$ B kinase (IKK) signalsome in a functional way. Interestingly, this IKK2 interaction is reportedly required for stimulus-dependent phosphorylation of other proteins in the FA complex.<sup>26</sup> FANCA also interacts with BRG1, a subunit of the SWI/SNF complex, and may regulate stress-induced chromatin remodeling.<sup>27</sup> Intriguingly, in FANCA mutant cells the molecular chaperone GRP94 is found in a BRG-1 associated complex but is not found in normal or complemented cells.<sup>27</sup> The significance of the phenomenon is unexplained but it is one of three specific examples in which aberrant or nonnative protein complexes are found in FA cells. In some instances (e.g., in the case of FANCC) there are aberrant signaling consequences that attend the formation of these complexes. Given that FANCA and FANCC both also associate with molecular chaperones,<sup>27-29</sup> it is quite possible that these and other FA proteins are primarily cochaperones that maintain the proper structural conformation (and state of activation or inactivation) of proteins that protect cells from environmental stresses. This theoretical model would fit with every binding phenomenon and biological function assay described to date for the FA proteins. For example, the core complex could create a scaffold for the cross-linking agent-induced activation of an ubiquitin ligase (possibly FANCL).<sup>30</sup>

### **FANCB**

One of the most recently discovered FA genes was identified using a proteomics approach.<sup>31</sup> FANCB was discovered by mass spectrometric analysis of a 95 kDa protein found in a multimeric FA complex. It has 10 exons, 7 of which are coding exons (3-10). The C terminus of the protein contains a putative bipartite nuclear localization signal and is present in nuclear extracts of normal cells but in mutant FA-A cells, FANCB is found, like FANCL, in the cytosol. That FANCB is required for FANCD2 monoubiquitination<sup>31</sup> confirms that FANCB is a functional member of the core complex.

The gene encoding this protein is located at Xp22.31 and subject to X-chromosome inactivation.<sup>31</sup> In the cases described to date there are different mutations in this gene including insertional frameshifts in exon 3 and 8, a deletional frameshift in exon 8, and a large 5' and promoter deletion.<sup>31</sup> It has been proposed that as a single active copy gene, the only X-linked FA gene, it may represent a vulnerability point for the genome of cells from obligate heterozygotes. An alternative view is that this protein too will prove to be multifunctional and required for cell survival. Indeed, studies of the methylation status of the gene showed that FANCB inactivation seems to be skewed toward the mutated allele.<sup>31</sup>

It is increasingly evident that the function of Fanconi proteins may be lost during multi-step carcinogenesis<sup>32-34</sup> but it is not clear whether the loss occurs early or late in carcinogenesis. Based on serial studies of cytokine responses of committed hematopoietic progenitor cells in humans with FA,<sup>35</sup> we have proposed that acquired loss of FA gene function cannot be an initiating event (such a cell would lose a competitive advantage) and that other somatic changes must occur first to protect such cells from the apoptotic consequences of FA loss.<sup>32,35,36</sup> Two recent findings have supported this model indirectly. First, apoptotic responses are indeed blunted in marrow cells of FA knockout mice with clonal evolution.<sup>37,38</sup> Second, the observation that in FANCB heterozygotes, X inactivation is skewed in favor of the mutated allele, indicates that the mutant cells are less fit to compete with the non mutant ones during development. A FANCB knockout mouse will be required to determine at which stage of development in heterozygotes the mutant FANCB cells are lost. There are likely other nonDNA damage related functions of the FANCB gene given that the affected males have classic FA phenotypes including bone marrow failure, but no data have been developed yet in support of this expectation.

### **FANCC**

This was the first of the FA genes to be cloned.<sup>13</sup> Ten to fifteen percent of FA cases can be accounted for by mutations of this gene.<sup>39</sup> Although there are a number of heterogeneous

**Table 2. Some nonFA FA-binding proteins**

FA Protein	NonFA Binding Partner	Functional Relevance
BRCA2	Rad51 <sup>77,78</sup>	Experiments demonstrating that FANCD1 mutant cells exhibited aberrant damage-induced Rad51 nuclear focus formation [79] led to identification of BRCA2 as a FA-D1 complementing gene. Mutations of BRCA2 that lead to FA disrupt the Rad51 binding region. <sup>80</sup>
FANCC	STAT1	FANCC facilitates activation of STATs by cytokines <sup>64</sup> and probably promotes survival of hematopoietic cells by doing so.
FANCC	hsp70	FANCC/hsp70 binding suppresses PKR activation <sup>28,29</sup> and thereby promotes survival of hematopoietic and embryonal cells.
FANCC	NADPH cytochrome p450 reductase <sup>130</sup>	Unknown.
FANCC	FAZF <sup>131,132</sup>	Unknown. Possible chromatin remodeling function. The relevance of FAZF is suggested by its consistent expression in primitive hematopoietic cells. <sup>131</sup>
FANCC	GRP94 <sup>133</sup>	Unknown. GRP94 has anti-apoptotic activity (93) and FANCC may facilitate that function.
FANCC	cdc2 <sup>49</sup>	Unknown but results of Kruyt et al <sup>134</sup> suggest the interaction may be functional
FANCC	GSTP1 <sup>54</sup>	Unknown
FANCA	BRG1 <sup>27</sup>	Unknown. Possible chromatin remodeling function (BRG1 is a subunit of the chromatin-remodeling SWI/SNF complex).
FANCA	Alpha spectrin and XPF <sup>135</sup>	Unknown.
FANCA	SNX5 <sup>136</sup>	Unknown. Possible co-chaperone effect of FANCA in SNX5-dependent receptor trafficking between organelles. <sup>136</sup>
FANCG	CYP2E <sup>137</sup>	Unknown
FANCD2	USP1	USP1 binds to and deubiquitinates FANCD2 and probably modulates the FA pathway in this way. <sup>89</sup>

mutations of this gene, two mutations account for most of the known FANCC mutations; the first in exon 1 (322delG) and the other in exon 4 (c.711 + 4A>T, also until recently termed "IVS4+4A-T" is a single base change in the fourth intronic base that results in deletion of exon 4). The 322delG mutation is hypomorphic for some FANCC functions,<sup>40</sup> likely because of a downstream reinitiation site that gives rise to a truncated protein.<sup>41</sup> The c.711 + 4A>T is unique to patients of Ashkenazi Jewish ancestry in which population the carrier frequency is 1/100.<sup>42,43</sup>

Because it was the first gene cloned more is known about the function of FANCC than any other FA gene. Many of the lessons learned about this protein will likely hold generally for most of the other members of the core complex as well. FANCC is a member of the core complex,<sup>44-46</sup> and binds to FANCE which facilitates nuclear translocation of FANCC.<sup>47,48</sup> At least one nuclear function of this protein is to facilitate the FANCD2/BRCA1 interaction in S phase and in cells exposed to cross-linking agents. Another might be to influence cell cycle control because the FAC protein (but not a mutant protein) coimmunoprecipitates with cyclin-dependent kinase, cdc2.<sup>49</sup> Other FANCC-interacting proteins are listed in Table 2.

### FANCC Is Required for Survival of Hematopoietic Cells

In mice, the most consistent FA phenotype is hypogonadism,<sup>50-52</sup> but in humans the single dominant early life-threatening feature of FA phenotype is hematopoietic failure that results from excessive apoptosis in hematopoietic cell populations including myeloid and erythroid progenitors.<sup>10,28,53-63</sup> The FANCC protein sets a high apoptotic threshold in normal cells and apoptotic responses of FA cells exposed to certain extracellular apoptotic cues (interferon gamma, tumor necrosis factor alpha, mip-1 $\alpha$ , fas ligand, and dsRNA) are exaggerated in vitro and in vivo.<sup>10,28,29,56,58,60,63-65</sup>

For the nuclear core FA complex to account for the hematopoiesis-specific anti-apoptotic function of FA proteins, it might serve to directly govern the expression of genes that encode intra- or extracellular survival factors specifically for hematopoietic cells. An alternative view, one better supported by experimental evidence, is that the FANCC proteins have functions independent of their capacity to form "the nuclear core complex" and that some of the FA proteins might even work entirely on their own (reviewed in ref. 10).

### FANCC and STAT Signaling

We determined more than a decade ago that FANCC is required for survival of hematopoietic cells.<sup>66</sup> Our results predicted that the FANCC protein either facilitated survival signals or suppressed apoptotic cues. It turns out that FANCC plays both roles. For example, FANCC is required for optimal activation of STAT molecules and the Jak kinase family member Tyk2, thereby facilitating cell survival and preserving interferon responses.<sup>10,64</sup> Recently it has been found that the disruption of this signaling pathway underlies a subtle T-cell defect in FANCC knockout mice.<sup>67</sup> The hematopoietic support function of CD4 cells is also perturbed (Fagerlie S, manuscript in preparation) emphasizing the multi-factorial nature of marrow failure in FA. That is, not only are myeloid and erythroid progenitor cells hypo-responsive to survival cues, lymphoid cells that support survival of progenitors are defective as well.

### FANCC, hsp70, and PKR

FANCC also governs the apoptotic threshold by suppressing pro-apoptotic signals. It suppresses the state of activation of the double stranded RNA dependent protein kinase, PKR, by binding to hsp70 (like a cochaperone) and facilitating the association of PKR and hsp70.<sup>28,29,56</sup> PKR exists in an inactive monomeric ground state in normal cells but is constitutively activated in FA-C cells.<sup>56</sup> As a result of this aberrant state of activation of PKR, exposure of mutant cells to IFN-gamma and TNF-alpha hyper-activates PKR and accounts for earlier observations that FA-C progenitor cells were hypersensitive to IFN-gamma<sup>68</sup> and TNF-alpha.<sup>58</sup> Indeed, PKR activity is now known to be increased in primary bone marrow cells of patients with FA-C, FA-A, and FA-G.<sup>29,65</sup> Interestingly, mutations of FANCC, FANCA and FANCG result in enhanced FANCC/PKR binding, and while it is unclear that this aberrant binding per se results in PKR activation (possibly by facilitating a structural change resulting in ground state PKR dimerization) the results do suggest that nonnative FA complexes can create serious pathophysiological consequences.<sup>65</sup>

### FANCC and Oxidative Stress

It has become increasingly clear that the FA proteins play a role in controlling cellular responses to a variety of extracellular challenges and stress; biotic, oxidative, and chemical. Results of an ongoing multi-institutional FA transcriptome consortium support this notion. Results of the study will be broadly shared with the scientific community in the future. There is also good biochemical evidence that FA proteins influence the states of activation of key mediators of cellular stress responses, including GSTP1 and ASK1.

Many investigators have long held that a fundamental defect of FA cells is intolerance to oxidative stress.<sup>69,70</sup> In support of this notion, Buchwald's group recently reported that FANCC enhances the function of GSTP1 in cells exposed to inducers of apoptosis.<sup>54</sup> GSTP1 is an

enzyme that detoxifies by-products of redox stress and xenobiotics. While FANCC does not directly interact with GSTP1, its influence on this molecule may play a central role in tolerance of extracellular cues of many kinds. Haneline and her colleagues recently observed ASK1 hyper-activation in H<sub>2</sub>O<sub>2</sub>-treated *Fancc*<sup>-/-</sup> cells and used gain- and loss-of-function analyses to confirm that the hypersensitivity of *Fancc* mutant cells to oxidative stress was mediated, at least in part, through altered redox regulation and ASK1 hyper activation.<sup>71</sup>

### **FANCC Is Multifunctional**

Studies on STAT signaling defects in FA cells have also permitted the development of structure-function studies that prove that FANCC is multifunctional. Specifically, a central, highly conserved domain of FANCC is required for functional interaction with STAT1 and conservative mutations of this domain interfere with STAT signaling functions of FANCC but these mutations have no impact on the capacity of the domain mutant to complement in MMC assays.<sup>40</sup> Another mutant (322delG) was unable to complement in MMC assays but did correct the STAT signaling defect. Interestingly, this mutation occurs in FA patients who have mild disease and mild hematopoietic defects.

### **Studies on FANCC and the Molecular Basis of Mosaicism**

Some patients with FA exhibit mosaicism in hematopoietic cells. These cases are ascertained by observing 2 subpopulations of lymphocytes, one sensitive and one resistant to cross-linking agents. Studies on FANCC structure have revealed mechanisms by which mosaicism occurs in such patients and can be used as a model for mosaicism in other complementation groups. Mosaicism can derive from recombination<sup>72</sup> or compensatory sequence alterations in *cis*.<sup>73</sup> Two patterns of recombination were described in haplotype analyses;<sup>72</sup> the first was a single intragenic crossover between the maternally and paternally inherited mutations and the second was likely gene conversion. In support of the hematopoietic survival function of FANCC, in the majority of the mosaic patients studied, the blood counts were only minimally suppressed.<sup>72</sup>

In summary, FANCC is a multifunctional protein that protects cells from the genotoxic consequences of cross-linking agent exposure by complexing with other members of the FA core complex to participate in the "linear pathway".<sup>21</sup> It also supports survival of cells in the ground state and cells exposed to a variety of biological cues that induce apoptotic responses. It does this by participating in at least three signaling pathways (STAT, PKR, and ASK signaling) at least in part by functioning very much in the same way a cochaperone would function. We believe that in time these nongenotoxicity functions of FANCC will be matched by like functions of the other FA proteins, some of which have domains (e.g., the TPR motifs in FANCG) that theoretically meet standards for cochaperone functionality.<sup>74-76</sup>

### **FANCD1 (BRCA2)**

Two observations converged to reveal the identity of the FANCD1 gene. First, it was known that BRCA2 binds Rad51,<sup>77,78</sup> and second that FANCD1 mutant cells exhibited aberrant damage-induced Rad51 nuclear focus formation.<sup>79</sup> These observations led to identification of BRCA2 as a FA-D1 complementing gene<sup>17</sup> and the confirmation that FA alleles of BRCA2 disrupt the Rad51 binding region.<sup>80</sup> This is not a common allele and because truly null mutations of BRCA2 are lethal in a murine model,<sup>81</sup> the mutations of BRCA2 that lead to FA are likely hypomorphic. The few FA-D1 patients identified to date have presented with early-onset malignancies of both hematopoietic and nonhematologic types.<sup>82</sup>

Monoubiquitinated FANCD2 seems to promote loading of BRCA2 into multimeric complexes on chromatin by binding to the C terminus of BRCA2.<sup>83</sup> Two separate sites on BRCA2 also interact with FANCG.<sup>84</sup> Using the I-SceI endonuclease to introduce a double-strand break at a specific chromosomal locus, Jasin's group found that BRCA2 mutant cell lines are recombination deficient.<sup>85</sup> Therefore, it is likely that these complexes are required for normal homology-directed DNA repair.

## **FANCD2**

The gene cloned by Markus Grompe's group at Oregon Health and Science University<sup>86</sup> led to the development of the "linear pathway model" for integration of FA protein function. FANCD2 associates with RAD51 and BRCA1 during S phase<sup>87</sup> but given the sheer size and likely complexity of these multimeric complexes, the association of these proteins per se is not sufficient to facilitate DNA repair by homologous recombination. Ubiquitination of FANCD2 requires the core complex but efficient ubiquitination also requires the ATR checkpoint kinase and RPA1.<sup>88</sup> Recently the deubiquitinating enzyme, USP1 was found to bind to FANCD2 (Table 2) and suppress expression of the ubiquitinated form.<sup>89</sup>

There is a good deal of uncertainty about the function of the monoubiquitinated form of FANCD2 and its functional relationship with BRCA1 and BRCA2. For example, there were conflicting reports on the influence of the FA pathway on Rad51 and BRCA2 focus formation in cells exposed to ionizing radiation. Ohashi et al<sup>90</sup> recently utilized BRCA2 and FANCD2 deficient cells and cells treated with siRNAs specifically targeting BRCA2, Rad51, and FANCD2 and developed data suggesting that FANCD2 does not have a direct role in BRCA2 and Rad51 associated homologous recombination repair after DNA damage.

This question still requires further evaluation because (1) survival assays were utilized (2) FA cells are hypersensitive to interferon (3) of the known capacity of some siRNAs to activate interferon responses,<sup>91</sup> and this pitfall was not experimentally ruled out.

## **FANCD2 and Diagnosis of Fanconi Anemia**

Inactivating mutations in all FA genes but BRCA2 lead to failure of accumulation of FANCD2 in damage-induced nuclear foci<sup>21</sup> and FANC alleles of BRCA2 interdict accumulation of Rad51 in such foci.<sup>79</sup> Therefore, although it is currently impractical, screening cells for FA lesions could involve microscopic assessments of damage-induced nuclear foci for FANCD2 and Rad51. FANCD2 immunoblots that distinguish the ubiquitinated and nonubiquitinated forms of FANCD2 might also be used to implicate inactivating mutations of any known FA gene except BRCA2.<sup>92</sup> In the context of an unambiguous FA phenotype (by MMC assays in the appropriate clinical setting), if FANCD2 is ubiquitinated the likely complementation groups would be FA-D1 or FA-J. BRCA2 mutants could be then identified using BRCA2 immunoblots which should reveal only truncated versions of the protein.

## **FANCE**

A rare FA gene, FANCE maps to 6p22-p21<sup>93</sup> and encodes a 536 amino acid protein that is part of the core complex<sup>15,94</sup> but plays a role in transporting cytoplasmic FANCC into the nucleus.<sup>48</sup> Mutations of FANCE include a 355C-T transition in the FANCE gene, leading to a gln119-to-ter (Q119X) nonsense change, a G-to-A change at position -8 in intron 5, resulting in false splicing and insertion of 6 nucleotides from intron 5, including an in-frame stop codon, and a 421C-T transition resulting in an arg141-to-ter (R141X) nonsense change.<sup>15</sup>

## **FANCF**

Discovered by complementation cloning, de Winter et al found that the FANCF gene is intronless and encoded a peptide with homology to the N terminus (RNA binding domain) of the prokaryotic RNA-binding protein ROM.<sup>14</sup> FANCF is largely nuclear and is a member of the core complex. Mutations of FANCF described to date are mostly deletions including a 23 base-pair deletion of nucleotides 230-252, a 47-bp deletion (349-395), a 2 bp deletion (484-485). Other mutations include a 16C-T transition resulting in a gln6-to-ter nonsense mutation, and a 327C-G transversion (yr109-to-ter nonsense mutation).

The protein encoded by this gene may function as a super-scaffold, organizing the formation of multimeric FA complexes. The C-terminus of FANCF interacts directly with FANCG and may facilitate the assembly of other FA proteins into the core complex (or other complexes for that matter).<sup>95</sup> The N-terminus of FANCF seems to stabilize the FANCA/FANCG interaction and is also required for binding of the FANCC/FANCE complex.<sup>95</sup>

### **FANCG**

By functional complementation of the Chinese Hamster ovarian cell line UV40, Liu et al<sup>96</sup> cloned XRCC9 a gene that conferred resistance to both hygromycin and mitomycin C. Later FANCG was shown to be identical to XRCC9<sup>97</sup> which had been localized to 9p13. The putative 622-amino acid nuclear and cytoplasmic protein is a member of the core complex<sup>46,98</sup> and is phosphorylated at serines 7, 385 and 387.<sup>99-101</sup>

Survival signaling functions of FANCG involve suppression (in collaboration with FANCC and FANCA) of the proapoptotic double stranded RNA-dependent protein kinase PKR.<sup>65</sup> In FANCG mutant cells, binding of FANCC and PKR is increased.<sup>65</sup> In keeping with the theme that nonnative interactions of FA proteins with signaling proteins in FA mutant cells contribute to pathogenesis, PKR activity is increased in bone marrow cells of patients with Fanconi anemia with mutations in the FANCC, FANCA, and FANCG genes.

There are numerous FANCG mutations. Most result in protein truncations<sup>102</sup> but the sites of mutation are not clustered. In 9 German FAG patients there was a 313G-T transversion in 8 of 18 (44%) mutated alleles.<sup>102</sup> In 7 Portuguese-Brazilian probands Auerbach et al<sup>103</sup> reported IVS8AS-2A-G. The same team also reported IVS11DS+1G-C in 7 French-Acadian probands, 1794-1803del in 7 European probands, and IVS3+1G>C (five Korean or Japanese probands) and suggest that the Portuguese-Brazilian, French-Acadian, and Korean/Japanese mutations were likely to have been present in a founding member of each of these populations. In black populations of sub-Saharan Africa, the incidence of FA is >1/40,000 and 82% of cases carry the same FANCG mutation (c.637\_643 del TACCGCC).<sup>104</sup>

FANCG protein is a member of the core complex, associates with FANCA and FANCF, and dimerizes with FANCA in cells exposed to oxidative stress.<sup>23</sup> FANCG has 6 or 7 (depending on the stringency of the consensus sequence definition) tetratricopeptide repeats,<sup>16</sup> elements that facilitate protein-protein interactions and, interestingly, are domains used by other cochaperones to bind to hsc70<sup>105</sup> and hsp90,<sup>76,106,107</sup> raising the distinct possibility that FANCG, like FANCC may integrate with heat shock responses as a cochaperone.

### **FANCH**

There is no FANCH gene. It was predicted from complementation group analysis carried out on cells later shown to be FANCA mutants.<sup>108</sup>

### **FANCI and FANCF**

Neither of these two recently identified genes has been cloned to date. That they exist is suggested by somatic cell fusion/complementation analyses and/or genetic data.<sup>12</sup> Using immunoblot analyses for FA core complex formation and FANCD2 mono-ubiquitination, Levitus et al demonstrated that both FA-I and FA-J cell lines formed a core complex, that FA-I cells were not capable of ubiquitinating FANCD2, and that FA-J cells did ubiquitinate FANCD2.<sup>12</sup> This placed the theoretical protein encoded by the mutated gene in FA-J cells "downstream" of the FANCD2 ubiquitination step.

### **FANCL**

This was the first of two FA genes (FANCB being the other) identified using a proteomics approach. Specifically, Meetei et al<sup>30</sup> identified, using mass spectrometry, a 43 kDa Fanconi complex associated protein as PHD finger protein-9 (PHF9). The 373-amino acid protein contains 3 WD40 repeats and a PHD-type zinc finger motif and is clearly a key component of the FA core complex. The protein has ubiquitin ligase activity and may play an important role in the monoubiquitylation of FANCD2.<sup>109</sup> In effect, the other members of the core complex may serve as a scaffold that permits the presentation of FANCL to FANCD2 and possibly to other proteins as well. In fact, taking into account the degree to which FANCD2 is conserved throughout evolution<sup>86</sup> it seems not sufficiently parsimonious that a complex of 7 FA proteins has evolved simply to facilitate one post translational change of FANCD2. Experiments are



now underway in our laboratory to identify other substrates that might be ubiquitinated by the FA complex.

The mutation described by Meetei et al<sup>30</sup> was a deletion of exon 11 that removed the part of the WD40 repeat and the entire conserved PHD finger. It arose by homozygous insertion of 177bp at the intron 10/exon 11 splice junction.

## FA Protein Complexes

### *The FA “Nuclear Core Complex”*

With the exception of FANCD2 and BRCA2, most of the FA proteins are thought to form a large multi-protein complexes.<sup>21,110-114</sup> Inactivating mutations of FANCA, FANCB, FANCC, FANCE and FANCG proteins reduce assembly, stability, and/or nuclear translocation of the multi-subunit FA protein “core” complex.<sup>46,114</sup>

One function of the core complex has been deduced by nicely-designed studies on isogenic FA mutant cell lines. These studies have clarified an emerging relationship between FANCA proteins and some functions of BRCA1, BRCA2 and Rad51. First, as mentioned above, certain BRCA2 (FANCD1) mutations can result in the FA phenotype (FA-D1)<sup>17</sup> and the mutations result in failure of Rad51 to localize in damage-induced nuclear foci.<sup>79</sup> Second, it seems clear that inactivation of any one of the core complex proteins interdicts formation of the complete FA complex, one consequence of which is reduced ubiquitinylation and BRCA1-colocalizing capacity of the nuclear FA protein FANCD2.<sup>21</sup> Therefore, accumulation of BRCA1 and Rad51 in damage-induced nuclear foci is required to protect cells from damage induced by cross-linking agents. Moreover, to accumulate in these foci, BRCA1 requires FANCA, B, C, G, and F dependent monoubiquitination of FANCD2.

What else might the FA complexes do in normal cells? Clearly the complex associates with chromatin in S-phase<sup>44</sup> and while there is no biochemical evidence that this is linked with a repair function, it is an appealing assumption in view of the importance of homologous recombination repair in S phase.<sup>115,116</sup> It is also specifically appealing in light of the linkage between the BRCA1/2 pathways and the FA pathway.

FA proteins, BRCA1, and BRCA2 are known to protect cells from cross-linking agent and oxidation induced genotoxicity.<sup>77,79,85,117-119</sup> Other proteins function in these ways as well, including ATR, Mre11, Tip60, NBS1, Rad51, Rad54, Rev3, Snm1, XRCC2, XRCC3, ERCC1, BLM, and Xpf, although none are associated with the disease Fanconi anemia. However, the precise biochemical functions of the FA complexes are unclear. Based on the current literature, it seems most likely that the FA proteins create scaffolds for assembly and proper folding of enzymes and multimeric complexes and that the client substrates of the FA proteins each function to protect the genome and set thresholds for responses to apoptotic cues. It is widely expected that these proteins are effectors of DNA repair (because of their known function in transcription coupled and homologous recombination repair,<sup>85,117,120</sup>) but biochemical studies have yet to confirm this. Moreover, as a reasonable parallel model, the BRCA1 and BRCA2 proteins exhibit other activities than their role in modulation of genotoxicity. They directly participate in transcriptional control,<sup>121-125</sup> cytoplasmic signal transduction,<sup>126</sup> and regulation of differentiation.<sup>124,127</sup> More work needs to be done to clarify the FA-BRCA1 relationships in functional terms.

### *Other Complexes*

The components of the FA complexes are increasing in number as it becomes clear that there is variability from method-to-method and from one subcellular compartment to another. Formation of complexes is a dynamic process that can be influenced by cell cycle phase, or by chemical or biological cues. Using chromatographic analyses Kupfer's group discovered that the FA “core complex” varies between subcellular compartments. The FA core complex exists in a 500-600 kDal cytoplasmic form, a larger 750 kDal cytoplasmic form seen only in mitosis,

a 2 MDal nuclear form, and a 1 MDal form bound to chromatin.<sup>45</sup> Interestingly, monoubiquitinated FANCD2 and BRCA2 also interact in chromatin fractions,<sup>83</sup> but because BRCA2 dependent RAD51 nuclear focus formation can occur independently of FANCD2,<sup>90</sup> the function of the D2/BRCA2 complex may have nothing to do with Rad51 focus formation.

As has been described above, the tendency of FA proteins to form large multimeric complexes has been exploited investigatively to discover new FA proteins. Using a global immunoprecipitation method, Meetei et al<sup>128</sup> identified 3 distinct multiprotein complexes associated with the Bloom's protein, BLM. One of these ("BRAFT") contained topoisomerase III- $\alpha$ , replication protein A and the core complex FA proteins. Other members of the complex ultimately proved to be FANCB and FANCL.<sup>30,31</sup>

In summary, most of the FA genes have been cloned now and the elucidation of the FA nuclear complex model by Garcia-Higuera et al<sup>21,46,129</sup> has been extraordinarily helpful in describing a unified vision of how mutations of these disparate proteins can lead to the same aberrant response to MMC. There is also a good deal of evidence that separate functions of the FA proteins account for the molecular pathogenesis of the hematopoietic defects and carcinogenesis but it is less clear whether these functions require participation of the other FA proteins or not. The most coherent functional view of FA proteins is that they act as cochaperones creating multimeric scaffolds that set response thresholds for environmental stress. In view of the emerging evidence that FA proteins affiliate with critically important tumor suppressor genes on the one hand, and participate in survival signaling pathways in hematopoietic cells on the other, studies on the function of these gene products have exciting promise for investigators interested in mammalian development, responses to biotic stress, carcinogenesis, hematopoietic control, and the immune response.

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