

## Review

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# The Fanconi anemia pathway of genomic maintenance

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**Abstract.** Fanconi anemia (FA), a recessive syndrome with both autosomal and X-linked inheritance, features diverse clinical symptoms, such as progressive bone marrow failure, hypersensitivity to DNA cross-linking agents, chromosomal instability and susceptibility to cancer. At least 12 genetic subtypes have been described (FA-A, B, C, D1, D2, E, F, G, I, J, L, M) and all except FA-I have been linked to a distinct gene. Most FA proteins form a complex that activates the FANCD2 protein via monoubiquitination, while FANCF and FANCD1/BRCA2 function downstream of this step. The FA proteins typically lack functional domains, except for FANCF/BRIP1 and FANCD1, which are DNA helicases, and FANCL, which is probably an E3 ubiquitin conjugating enzyme. Based on the hypersensitivity to cross-linking agents, the FA proteins are thought to function in the repair of DNA interstrand cross-links, which block the progression of DNA replication forks. Here we present a hypothetical model, which not only describes the assembly of the FA pathway, but also positions this pathway in the broader context of DNA cross-link repair. Finally, the possible role for the FA pathway, in particular FANCF and FANCL, in the origin of sporadic cancer is discussed.

Keywords: Fanconi anemia, cancer, genetic predisposition

## 1. Carcinogenesis

Cancer arises by multiple alterations in the genome. Two general classes of genes may be distinguished according to their role in cancer formation: gatekeeper genes and caretaker genes. The first class is comprised of two types of genes: oncogenes and tumor suppressor genes, which are directly involved in the control of cellular proliferation. Control of cellular proliferation can be achieved by two separate mechanisms, by regulating cell proliferation and by regulating the occurrence of cell death. In normal tissues the rate of cell birth equals the rate of cell death, resulting in homeostasis. Previously, tumor development was thought to be predominantly caused by an increased rate of cell birth. Today, it is recognized that tumors arise due to an imbalance between cell birth and cell death. Genes that promote cell birth or cell proliferation are called

proto-oncogenes. These proto-oncogenes, which are highly conserved in evolution, are important regulators of normal cell proliferation and differentiation. When a mutation alters the structure of these proto-oncogenes, thereby making them constitutively active or altering their expression levels or sites of expression, these proto-oncogenes become oncogenes. Oncogenes are responsible for uncontrolled cell growth by increased cell birth. They perform the same function as proto-oncogenes but are beyond control or out of control. Just as proto-oncogenes are thought to regulate normal cell proliferation, the action of tumor suppressor genes is to constrain cell growth in normal cells. Mutations that inactivate tumor suppressor genes therefore liberate the cell from growth constraints imposed by these genes, which normally control cell death or cell cycle arrest. A combination of activated oncogenes and inactivated tumor suppressor genes generally characterizes a cancer cell.

The second class of genes involved in cancer formation are the caretaker genes or stability genes. These genes are not directly involved in control of

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cell growth, but are responsible for maintaining the integrity of the genetic information in the cell. DNA can become damaged by both endogenous as well as exogenous factors. Each cell has a number of different pathways that repair specific types of DNA damage. For example, the nucleotide excision repair (NER) pathway is involved in repairing damaged bases such as thymine dimers induced by UV light [41]. Inactivation of these DNA repair genes will result in loss of an effective DNA repair system, which may lead to a higher mutation rate. In other words, stability genes keep genetic alterations to a minimum and when inactivated, cells become genetically unstable, i.e., genomic mutations will occur at a higher rate. Since cancer development requires multiple mutations, a higher mutation rate is an important prerequisite in cancer development. Important targets for mutagenesis are gatekeeper genes (proto-oncogenes and tumor suppressor genes). Current understanding of the process of carcinogenesis is in line with the hypothesis that a cell needs to acquire a high mutation rate or mutator phenotype in order to become at risk for developing into a malignant cancer cell [91]. In this process cells not only acquire mutations, but also gain and lose chromosomes and chromosomal regions resulting in aneuploidy and chromosomal disorganization [35].

Inherited mutations in stability genes may lead to two types of apparent modes of inheritance. In dominantly inherited conditions only one mutated gene is inherited and the other allele must be inactivated before an accelerated mutation rate is achieved (BRCA1/2, for predisposition for breast/ovary cancer, and DNA mismatch repair genes, for hereditary nonpolyposis colorectal cancer). In recessively inherited diseases (such as xeroderma pigmentosum, Bloom syndrome, and Fanconi anemia), both mutated genes are inherited from the parents and all the cells of the body are genetically unstable. Therefore in these syndromes the acquisition of additional mutations in oncogenes and tumor suppressor genes is much more likely. After a mutation in an oncogene or tumor suppressor gene, the cell starts to proliferate abnormally and has become prone to develop into a cancer cell.

Accordingly, a germline mutation in any of these genes will lead to cancer predisposition, that is, cancer arises with high probability and often multiple tumors will develop at an earlier age than in individuals who develop sporadic tumors [152,153]. In this review we will focus on the stability genes that underly the inherited syndrome Fanconi anemia.

## 2. Fanconi anemia

### 2.1. Clinical characteristics

FA is a rare recessive disease with both autosomal and X-linked inheritance and has an estimated heterozygous carrier frequency of around 1 in 300, based on the incidence of affected individuals in the State of New York in 1971 [95,129,138]. The incidence of FA varies among ethnic populations. In some populations the incidence is higher due to a founder effect or where consanguineous marriages are common [122,151].

Fanconi anemia was first reported in 1927 by the Swiss paediatrician Guido Fanconi [37]. He described a family with three male children with pancytopenia and birth defects. Diagnosis of FA patients has been based on this description for many years. Subsequently FA became known as an autosomally inherited disease with diverse clinical symptoms, such as skeletal malformations (hypoplasia of the thumbs and radial hypoplasia), hyper-pigmentation in the form of café-au-lait spots and/or areas of hypopigmentation, small stature and low birth weight (sometimes related to growth hormone deficiency) [156]. Renal abnormalities are present in about one third of the FA patients and include renal aplasia or hypoplasia, horseshoe kidneys or double ureters. Urogenital malformations are also common, that is undescended testes in males and uterine abnormalities in females; additionally, hypogonadism and infertility are common in both [146]. The most important clinical features of FA are in fact haematological, as FA is a bone marrow failure syndrome. FA patients show a high incidence of aplastic anemia, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). At birth the blood counts are usually normal, the first symptom of bone marrow failure being macrocytosis, followed by thrombocytopenia and neutropenia [18,146]. Pancytopenia usually does not set in until about 7 years of age [9,18]. The majority of the patients eventually die of AML, for which they have a strongly increased risk (about 1000 times) [121]. Those patients that do survive the haematological malignancies have to deal with an array of solid tumors, mainly squamous cell carcinomas of the head and neck area, for which they show a similarly increased risk [5,6]. Bone marrow failure in combination with cancer proneness reduces the average life expectancy to about 15 to 20 years [24,153].

## 2.2. Cellular characteristics

The first major discovery characterizing FA at the cellular level was that FA lymphocytes exhibit excessive spontaneous chromosomal instability. This instability was seen in the form of chromatid gaps and breaks, and various chromatid interchanges. Such interchanges involve heterologous chromosomes [127,128]. This chromosomal instability feature is now thought to be responsible for the cancer susceptibility of FA patients.

## 2.3. Cross-linker hypersensitivity

Another important discovery has been the hypersensitivity of FA cells to the clastogenic effect of cross-linking agents, such as mitomycin C, diepoxybutane and cisplatin [10,62,126,161]. Such bifunctional alkylating agents generate DNA cross-link damage, which greatly exacerbates the chromosomal instability typically seen in FA cells. This specific trait is now widely used in the diagnosis of FA patients. Kano and Fujiwara (1981) claimed that FA cells also show an increase in the formation of sister chromatid exchanges (SCE) after MMC treatment, suggesting a role for the FA pathway in homologous recombination repair [71]. However, more recent evidence shows that SCE formation in FA cells is similar as in normal cells after MMC treatment (Joenje and Oostra, unpublished data) casting doubt over the original data by Kano and Fujiwara.

Cross-linking agents or bifunctional alkylating agents generate predominantly intrastrand cross-links, which connect bases within the same strand. However, the same agents also generate interstrand cross-links, in which the antiparallel DNA strands in a double helix are connected [6]. Interstrand cross-links represent only a small fraction of the lesions formed by DNA cross-linking agents (5–13% for MMC), but are thought to be the most toxic lesion, since they inhibit DNA strand separation and therefore block DNA replication, transcription and segregation [32,159]. The sensitivity seen in FA cells seems to be restricted to interstrand cross-links, as they do not show sensitivity to UVC, a typical intrastrand cross-linker, monofunctional alkylating agents such as EMS, or to ionizing radiation [34,42,69,161]. However, there is some dispute about the latter agent, since some papers do report hypersensitivity of FA cells to ionizing radiation [15,21,47,139].

## 2.4. Cell cycle abnormalities

The process of cell division is tightly regulated by cell cycle checkpoints, since an abnormal cell cycle may lead to malfunctioning daughter cells and uncontrolled growth. At the G1 cell cycle checkpoint, the cell determines whether all the preparative stages for DNA synthesis have been completed correctly and whether the cell is ready to proceed into the S phase. During the DNA replication phase an S phase checkpoint ensures that DNA synthesis can be successfully completed, before cells can progress to the G2 phase. If DNA damage has occurred such damage will have to be repaired before the cell can proceed into the G2 phase. This is ensured by this checkpoint, although certain types of DNA damage can also be repaired during the G2 phase. The G2/M checkpoint ensures that the cell is prepared and ready for cell division in the M phase. If cells are delayed or arrested at a certain point in the cell cycle, this will endure for as long as necessary to repair all the damage. Only then will the cells be able to pass the checkpoint. If the damage cannot be repaired, the cells may go into apoptosis.

FA cells generally exhibit an abnormality in cell cycle distribution in that they show an increased proportion of cells with 4N DNA content. This indicates that in FA cells a G2/M or late S phase delay or arrest occurs [36,77]. This prolonged G2 phase or G2 cell cycle arrest is further enhanced after treatment with cross-linking agents [68]. Since p53, a well known tumour suppressor is involved in G1/S and G2/M cell cycle transitions, FA cells may be abnormal through a direct interaction between an FA protein and p53. However, FA cells show a normal p53 protein induction after treatment with MMC or other DNA damaging agents. Moreover, p53-dependent apoptosis is normal in FA cells following treatment with MMC. Furthermore, FA cells and normal cells behave similarly in cell cycle progression from G1 phase to S phase [74,77,79]. Taken together, these results suggest that p53 induction and G1/S transition is normal in FA cells and G2/M arrest does not occur through a direct interaction between an FA protein and p53.

Studies have shown that the type of damage that is induced determines the type of repair that is called upon and at which stage of the cell cycle repair is occurring. Gamma-irradiation during G2 causes double strand breaks (DSB), which causes a G2/M cell cycle arrest [57]. However, induction of interstrand cross-links during G2 stage of the cell cycle does not result in chromosomal breakage or a G2/M arrest until the next

cell cycle. The same holds true for interstrand cross-links induced during the G1 stage, which does not result in a G1/S cell cycle arrest. In both FA and wild type cells chromosomal breakage and a cell cycle arrest is only observed, after the cells have undergone DNA replication [2,3]. This indicates that DNA replication is required in wild type and FA cells to induce a G2/M arrest and chromosomal breakage. Since arrested FA cells have a 4N DNA content, it is unlikely that the FA pathway is involved in a checkpoint that induces early- or mid S phase arrest. More likely is that the G2 arrest observed in FA cells represents incomplete DNA replication and an arrest in late S-phase, rather than a G2/M arrest. In contrast to wild type cells, FA cells take up to three times longer to clear this arrest, indicating that the FA proteins are somehow involved in resolving interstrand cross-links during late S-phase [2]. Furthermore, FA cells do not stop DNA replication after interstrand cross-links are induced, as is seen in wild type cells [22,124].

Overall, it seems that FA cells have a defect during DNA replication in the S phase of the cell cycle, either due to defective repair of DNA damage induced during the S-phase or to damage-resistant DNA synthesis, or both, which ultimately results in a late S-phase delay rather than a G2 or G2/M delay.

### 3. Genetic basis of Fanconi anemia

#### 3.1. Heterogeneity in Fanconi anemia

Fanconi anemia is highly heterogenous, both clinically and genetically. Fanconi anemia can be subdivided in no less than twelve genetic subtypes or complementation groups, FA-A to FA-M [33,64–66,84,94,96,136,137]. Each group is connected to a distinct disease gene. Not all groups are equally represented. Most patients belong to group FA-A (approximately 60–80%), followed by groups FA-C and FA-G. The four smallest groups are FA-L and FA-M with both only a single patient identified so far, followed by the FA-I and FA-B groups with four patients per group (Fig. 1).

#### 3.2. Complementation analysis

Complementation groups were defined by cell fusion studies and complementation analysis. In this procedure two immortalized FA patient lymphoblastoid cell lines are created and marked with a selection marker, as first described by Duckworth-Rysiecki et

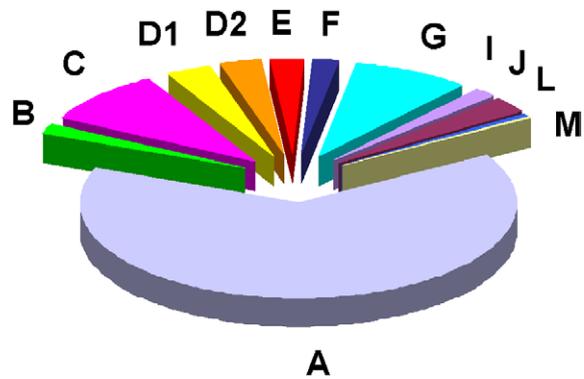


Fig. 1. Relative prevalences of complementation groups in FA. Results are based on the first 248 FA families classified by the European Fanconi Anemia Research Program (1994–2003). This number includes the reference cell lines for groups A, B, C, D1, and D2. The absolute numbers per group were as follows: A: 159; B: 4; C: 23; D1: 8; D2: 8; E: 6; F: 5; G: 21; I: 4; J: 8; L: 1, M: 1. Figure reproduced, with permission, from the thesis of M. Levitus.

al. in 1985, Strathdee et al. in 1992 and further developed by Joenje et al. in 1995 [33,65,137]. Cell lines are fused pairwise to result in heterokaryons, including viable hybrids that possess 4N DNA content. This fusion hybrid is then tested for MMC sensitivity in a growth inhibition assay. If the fusion partner cell lines were defective in the same gene, the hybrid is still sensitive and the partners are said to belong to the same complementation group. If, however, both cell lines are defective in different genes, the defect will be corrected in the hybrid to result in wild type sensitivity to MMC and the cell lines are said to belong to different complementation groups (Fig. 2). By applying this method to a large number of FA patients, a total of ten complementation groups could be defined (FA-A to FA-J, including the retracted FA-H group). Although this procedure has been quite effective in defining complementation groups for FA, the analysis has some pitfalls.

One of these is false non-complementation. In this case a fusion hybrid shows a FA-like phenotype and both cell lines are therefore classified as belonging to the same complementation group. However, some fusion hybrids can lose chromosomes, which can lead to misclassification if the complementing chromosomes are lost. Therefore, non-complemented hybrids should always be tested for chromosome loss to avoid such misclassification. Although this minimizes the chance of wrong classifications, this approach does not completely rule out misclassification, as illustrated by the analysis of patients in the FA-D group. This group was composed of four patients and a textbook example of non-complementation of fully tetraploid

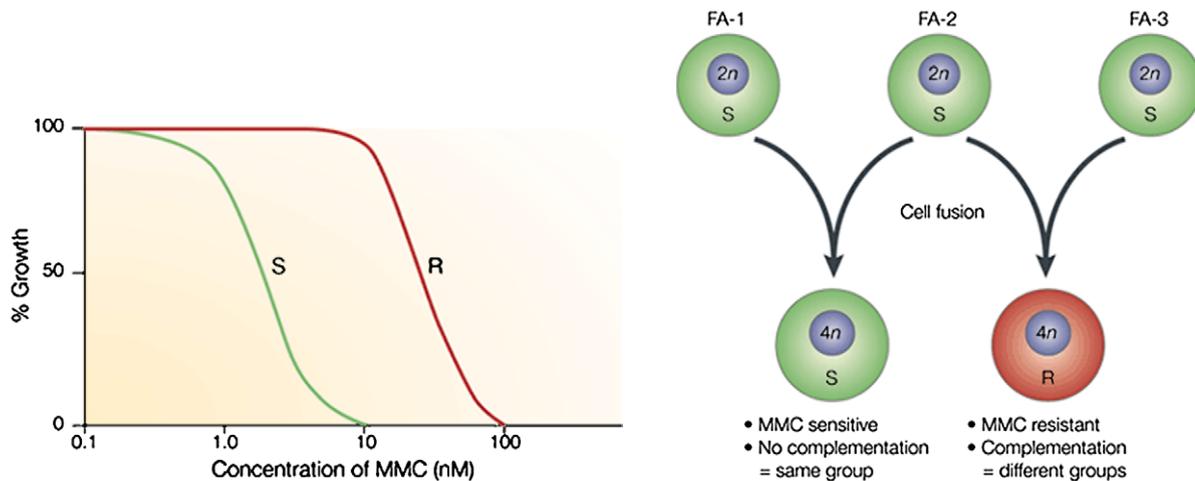


Fig. 2. Complementation analysis in FA. If two patient cell lines are fused together, the hybrid cell can either become sensitive (S, in which 50% growth reduction (IC<sub>50</sub>) is seen at a concentration below 10 nM MMC) or resistant (R, with an IC<sub>50</sub> value of more than 10 nM of MMC). An MMC-sensitive cell line indicates that both patient cell lines are defective in the same FA gene and therefore belong to the same complementation group (green hybrid cell). If, however, a hybrid cell becomes resistant to MMC, the result indicates that the cell lines are defective in different FA genes, so that the MMC sensitivity is corrected (red hybrid cell). (Figure reproduced with permission from *Nature Reviews Genetics* (Joenje and Patel) [67] © (2001) Macmillan Magazines Ltd.)

hybrids [64,65]. Eventually this single group turned out to be composed of two different complementation groups, with two cell lines mutated in one gene (*FANCD1/BRCA2*) and two cell lines mutated in another gene (*FANCD2*) [58,145]. The opposite can also occur, that is “false” complementation. In this case a fusion hybrid shows a wild type phenotype, normally indicating that both cell lines belong to different complementation groups. However, in some rare cases both cell lines may belong to the same complementation group instead. This happened with the FA-H group which was represented by a single cell line [66]. Finally, this FA-H cell line turned out to have mutations in the *FANCA* gene and therefore belonged to the FA-A complementation group; as a result, group H was withdrawn and new, more stringent, criteria for the definition of complementation groups were proposed [64].

After the genes for some of the complementation groups were identified, alternative methods became available to classify FA patients according to complementation group. One method involves the introduction of a cDNA into the unclassified cell line. This can be accomplished by inserting the cDNA into a retroviral or episomal expression vector and transfer into the cells by transduction or transfection methods. However, this procedure is only useful if the defective gene of the particular complementation group is known. Another alternative is to detect the presence or absence of the FA protein of interest via a Western blot procedure.

The advantage of this method is that it is relatively quick, but is dependent on the availability of a suitable antibody to detect the protein. Furthermore, some pathogenic missense mutations still allow normal levels of full-length protein to be produced. Results from Western blot procedures are therefore often inconclusive, but could provide useful indications how to proceed to make a final assignment. The ultimate way to classify a FA patient is by identifying pathogenic mutations in both alleles of a FA gene. Currently, only for the FA-I complementation group the disease gene has not been identified yet.

Identification of new FA genes has been accomplished by complementation cloning, positional cloning or protein complex purification. Positional cloning is usually based on large families belonging to the same complementation group. In the case of consanguineous families finding a single homozygous region within the entire genome identical in all patients (homozygosity mapping) reveals the approximate location of the defective gene and screening candidate genes within the region for mutations in the patients will finally identify it. However, by using microcell-mediated chromosome transfer, a combination of positional cloning and complementation cloning, a chromosome or part of a chromosome can be identified harboring the defective gene using a single cell line. In this procedure, a complete or partial human chromosome is introduced into the FA cell line. This hybrid can then

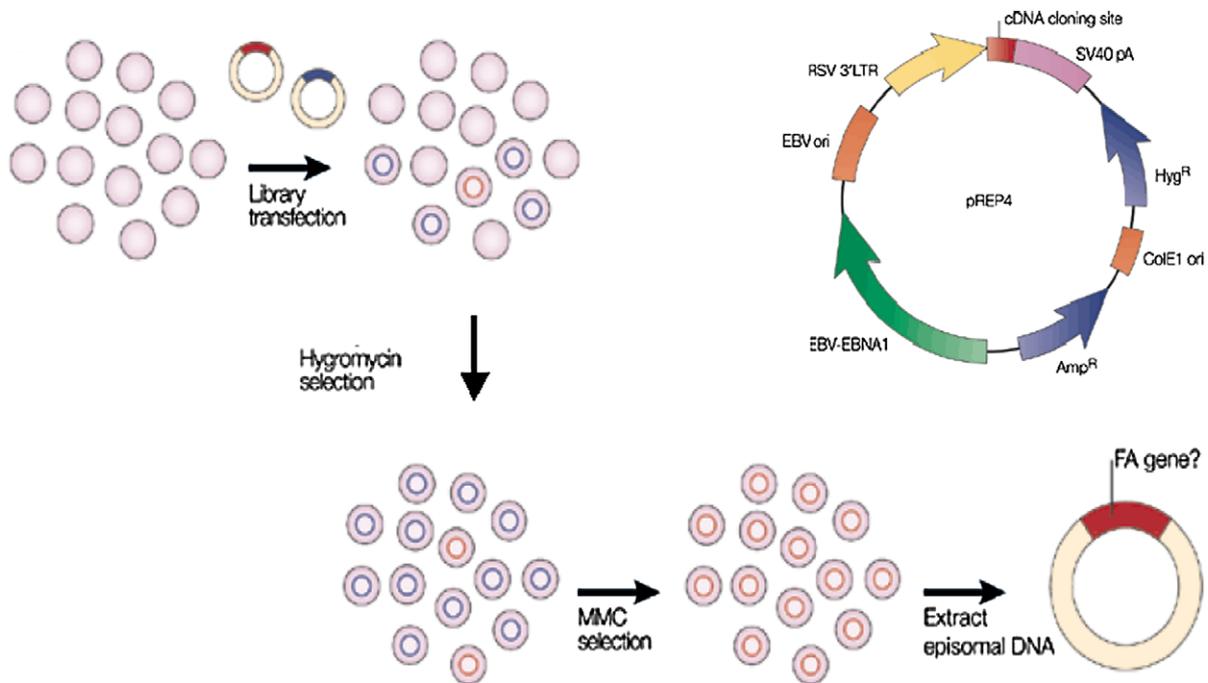


Fig. 3. Complementation cloning in FA. Different cDNAs, contained in a library derived from wild type human lymphoblasts, are cloned into the pREP4 vector. This vector is introduced into a MMC-sensitive FA cell line. First, a hygromycin selection is performed to select for those cells that have taken up a vector. Secondly, this cell population is selected for those cDNAs that confer MMC resistance. This MMC resistant cell population is likely to contain the FA gene defective in the original cell line. The different cDNAs in this population, all candidate-FA genes, are then extracted and examined. Detection of mutations in a candidate gene in the patient provides final proof that a new FA gene has been identified. (Figure reproduced with permission from *Nature Reviews Genetics* (Joenje and Patel) [67] © (2001) Macmillan Magazines Ltd.)

be tested for complementation of its MMC sensitivity. In this way the presence of a complementing piece of chromosome may be correlated with complementation of the cellular defect. As soon as the critical region has been narrowed down sufficiently, subsequent screening of candidate genes in the region will reveal the defective gene. Protein complex purification is based on the precipitation of FA (core complex) proteins and identification of all interacting proteins. These interacting proteins are candidate-new FA proteins and mutation screening in patient cell lines may reveal whether a new FA gene and consequently a new FA complementation group has been found. The various methods used to identify FA genes are discussed in more detail below.

### 3.3. Cloning FA genes

The general idea that each complementation group would be defective in a single disease gene is supported by the actual situation so far; presently, only the *FANCI* gene has not been identified. FA gene cloning

started with the discovery of the *FANCC* gene in 1992 using complementation cloning (Fig. 3) [136]. Complementation cloning is also referred to as functional cloning, since it is based on the principle that introduction of the wild type variant of the defective gene complements the phenotypic defect in the cell line. This phenotypic trait makes it possible to select for cells that have been complemented and therefore phenotypically corrected. For FA, this phenotypic defect is the extreme sensitivity to cross-linking agents, such as MMC. Introducing a cDNA expression library into the cells and subsequent selection for those cells that have taken up cDNAs and a final selection on MMC selects for only those cells that are corrected for the cellular phenotype. These resistant cells are theoretically complemented with the cDNA or gene that is defective in the original cell line. Identification of that cDNA and mutation screening in the patient must prove if a new FA gene has actually been identified or not.

The method seems quite straightforward but does have some prerequisites: first, the cDNA library used must contain the relevant cDNA with its open read-

ing frame intact; second, the FA protein should not be toxic when overexpressed; third, the cell line should be able to take up the DNA, which is often a limiting factor, since human lymphoblastoid cell lines are usually reluctant to take up exogenous DNA; fourth, the FA cell line should not revert spontaneously at a significant rate to a MMC-resistant state. In spite of these limitations a number of genes have been cloned using this method. After the initial identification of *FANCC* this same approach was used to identify *FANCA* in 1996 [89], although next to identification through complementation cloning, *FANCA* was independently identified by positional cloning as well [1]. Complementation cloning continued to be very successful in the years thereafter, when three more genes were identified. *FANCG* was found in 1998, followed by *FANCF* and *FANCE* in 2000 [28,29,31].

*FANCD2* and *FANCI/BRIP1* were cloned by a different method. For these genes a combination of positional and complementation cloning was used, a laborious but rather robust procedure to identify new genes. First, microcell-mediated chromosome transfer identified chromosome 3 as the complementing chromosome for *FANCD2*. Next, positional cloning using partially deleted chromosomes 3 revealed a map location at 3p22–26, followed by the identification of the defective gene in this group by screening candidate genes for mutations [145,162]. *FANCI/BRIP1* was cloned using the same approach, but the other way around. First, linkage analysis revealed chromosome 17 as the most likely chromosome to harbor the *FANCI* gene, which was confirmed by microcell-mediated chromosome transfer. Next, *FANCI/BRIP1* was screened as a candidate gene and was found to carry mutations in all FA-J patients examined [85]. *FANCI* was also independently identified using slightly different methodology [86,88].

*FANCD1* was identified by the candidate gene approach. Mutation screening of *BRCA2* in FA-D1 patients revealed biallelic pathogenic mutations, indicating that *BRCA2* is in fact the *FANCD1* gene [58].

For the last genes cloned so far, *FANCL*, *FANCB*, and *FANCM*, an approach other than complementation cloning or positional cloning was used [94–97]. The identification of these genes was based on protein complex purification. By identifying proteins that bind to the *FANCA* protein, the common complex proteins *FANCC*, *FANCG*, and *FANCF* were found, in addition to a number of unidentified proteins. These were termed FAAPs, for Fanconi Anemia Associated Proteins, followed by a number indicating their ap-

proximate molecular weight. The *FANCL* protein was initially termed FAAP43, since it has a molecular weight of 43 kDa. Western blot experiments revealed a single FA patient whose lymphoblasts missed *FANCL* protein expression. Mutation analysis then confirmed that this patient was indeed mutated in the corresponding gene, termed *FANCL*, so that a new FA gene and complementation group were discovered at the same time. Next to *FANCL*, three other proteins were co-precipitated with *FANCA*, namely, FAAP95, FAAP100, and FAAP250. By using the same approach as used to identify *FANCL*, FAAP95 was identified as *FANCB*, while FAAP250 was identified as *FANCM*. This left only FAAP100 unidentified, which could represent a new FA gene and complementation group. The FA-L and FA-M complementation groups are the only groups to date that are represented by a single patient and in which the genes were found before the complementation groups were identified. Table 1 summarizes all currently known FA genes, how they were identified, their chromosomal locations, and features of their protein products.

#### 4. Proteins acting upstream of *FANCD2* activation

##### 4.1. *FANCA*, *FANCB*, and *FANCG*

*FANCA* was first found to interact with *FANCC* using co-immunoprecipitation experiments [30,44,75,78]. A direct interaction between *FANCA* and *FANCC* was not found using yeast two-hybrid analysis, indicating that *FANCA* and *FANCC* might be part of the same complex, but do not bind directly to one another [59,93].

A direct interaction was detected between *FANCA* and *FANCG* by co-immunoprecipitation experiments and yeast two-hybrid analysis [30,46,51,59,73,93,119,154]. This interaction is present in all FA complementation groups, indicating that formation of the *FANCA-FANCG* subcomplex is one of the initial steps in the FA core-complex assembly [30,154].

*FANCA* has both a nuclear and a cytoplasmic localization. An N-terminal bipartite NLS sequence, in combination with sequences at the C-terminus are needed to assure proper nuclear localization of the protein [78,87,89]. Based on the predominant cytoplasmic localization of *FANCA* in FA-B cells and the lack of phosphorylated *FANCA* in cells of all complementation groups (except in FA-D1), it was long thought that *FANCB* might be the kinase responsible

Table 1  
 Characteristics of all currently known FA genes and products

Gene	Identification method	Location	CDS size (bp)	Protein size (kDa)	Motifs or domains
FANCA	CC/PC	16q24.3	4368	161	Bipartite NLS and Leucine zipper
FANCB	PP	Xp22.31	2579	95	NLS
FANCC	CC	9q22.3	1674	65	–
FANCD1/BRCA2	CS	13q12.3	10256	384	8 BRC motifs for RAD51 binding
FANCD2	PC	3p25.3	4415	155/162	Monoubiquitination at K561
FANCE	CC	6p21.2–21.3	1610	63	2 NLS
FANCF	CC	11p15	1124	42	Adaptor protein
XRCC9/FANCG	CC	9p13	1868	70	7 TPR motifs for protein binding
FANCI/BRIP1	PC, CS	17q22	3749	141	7 helicase motifs, ATP dependent 5' → 3' helicase
FANCL	PP	2p16.1	1127	43	Ring finger motif typical for E3 ubiquitin ligases
FANCM	PP	14q21.3	6044	250	Endonuclease and helicase domain

CC, complementation cloning; PC, positional cloning; PP, protein association; CS, candidate gene approach.

for nuclear accumulation of phosphorylated FANCA [30,166]. However, FANCB does not contain a protein kinase domain, making it very unlikely that FANCB is responsible for FANCA phosphorylation. Additionally, FANCA is not phosphorylated in all complementation groups that affect the core complex and therefore it seems more likely that the whole core complex is involved in this modification or in stabilization of the modified form of FANCA.

In the absence of FANCB, FANCL is also not detected in the nuclear compartment. FANCB was found to directly interact with FANCL in the mammalian two hybrid assay, suggesting that FANCB may aid transport of FANCL into the nucleus, through its NLS sequence (Medhurst et al., unpublished data). Since FANCA is also needed for the nuclear accumulation of FANCL and the FANCB/FANCL subcomplex does bind FANCA, it is possible that FANCB and FANCL enter the nucleus together with FANCA and FANCG [94] (Medhurst et al., unpublished data). Alternatively, FANCA/FANCG and FANCB/FANCL might enter the nucleus separately through their NLS sequences and form a subcomplex inside the nucleus, rather than in the cytoplasm. This larger complex is then needed to maintain nuclear localization.

FANCG, like FANCA, is found in the nucleus as well as in the cytoplasm [154]. The protein has seven motifs known as tetratricopeptide repeat motifs (TPR), which are 34-nucleotide sequences thought to be involved in protein-protein interactions. Disrupting these motifs leads to reduced FANCA binding, suggesting

that the TPRs are important for the interaction with FANCA [16]. The interaction between FANCA and FANCG is direct and based on binding of FANCG to the N-terminal NLS sequence of FANCA [45,76,154]. Apart from binding to the FANCA protein, the C-terminal part of FANCG is thought to bind the FANCC protein, since deletion of the last 39 amino acids makes FANCG unable to recruit FANCC into the complex [76]. However, no direct interaction has been found between FANCG and FANCC, although the proteins belong to the same complex [30,46,51,93]. It seems more likely that FANCF forms the bridge between FANCG and FANCC since the C-terminus of FANCF binds FANCG and the N-terminus of FANCF is able to bind FANCC and FANCE [83].

Apparently, FANCG also binds directly to FANCD1/BRCA2 at the N- and C-termini, around the BRC repeats [61]. The precise implications of this interaction are not known at present, but combined with results indicating a direct binding of FANCA and FANCI to BRCA1, FANCD2 to FANCD1/BRCA2, and the colocalization of FANCD2 and BRCA1 in DNA damage-inducible nuclear foci, these data strengthen the notion that the FA pathway is closely linked to the BRCA pathway and possibly involved in repair through homologous recombination (HR) [38,47,85,158].

#### 4.2. FANCC and FANCE

At first, FANCC was thought to have a cytoplasmic function, due to its predominantly cytoplasmic loca-

tion in cell fractionation and immunofluorescence experiments [165,168,169]. Later a small proportion of the protein was also detected in the nuclear compartment [55]. Next to the debated FANCA/FANCC interaction, FANCC was also found to interact with other FA proteins, especially with FANCE, which is a direct interaction [51,93,111].

FANCE, a predominantly nuclear protein, is thought to promote nuclear localization of FANCC. Co-expression of the FANCE protein with FANCC, promotes FANCC to be targeted into the nucleus. In the absence of FANCE, lower levels of FANCC were seen in cytoplasm and nucleus, which could be restored after re-introduction of the FANCE protein. Additionally, C-terminal FANCC mutants are unable to interact with FANCE and also lose the ability to co-localize with FANCE in nuclear foci [111,140].

As FANCC presumably does not interact with the cytoplasmic complex of FANCA, -B, -G and -L and since this protein lacks an apparent NLS sequence, nuclear localization of FANCC might partly depend on its relatively small size, which through diffusion transport may be responsible for moving FANCC into the nuclear compartment. However, nuclear localization of FANCC could also be due to a carrier protein, for example FANCE, or a still unidentified NLS sequence. FANCE enhances localization of FANCC in the nucleus, possibly by binding and retaining FANCC in the nucleus. If no FANCE is present, for example in FA-E cells, all FANCC resides in the cytoplasm [51,55,111,140].

FANCE has also been shown to interact with FANCA and FANCG in co-immunoprecipitation experiments [140]. However, only a weak direct interaction of FANCE with FANCA and FANCG was detected by yeast two-hybrid analysis, indicating that this is possibly an indirect interaction, which is dependent on other proteins [93]. A direct interaction between the C-terminus of FANCE and the N-terminus of FANCD2 has been found, thereby linking FANCD2 to the FA complex [51,111]. FANCC also interacts with a number of unrelated proteins, such as cyclin-dependent kinase *cdc2*, Hsp70 and PKR. The latter two interactions suggest a role for FANCC in supporting hematopoietic cell survival, since Hsp70 suppresses PKR activity, which is involved in this process [112]. FANCC has also been reported to be involved in apoptosis and tumorigenesis, since in FANCC/p53 double mutant mice, malignancies are formed more rapidly than in the single mutant mice and are of the same type as those found in FA patients. These results suggest an in-

teraction of FANCC with p53, possibly implicating involvement in processes regulated by p53. Another possibility is that these malignancies manifest more readily when p53 control is disrupted. Recently, FANCC and p53 were both suggested to regulate G2 checkpoint control, again linking FANCC to cell cycle control [39,40].

#### 4.3. *FANCF*

FANCF shows homology to the prokaryotic RNA-binding protein ROM, which suggested a possible function for FANCF in RNA binding [29]. However mutational disruption of the ROM motif does not appear to alter FANCF's functional activity in a complementation assay [83]. Immunoprecipitation studies and cellular fractionation studies have provided evidence for a mainly nuclear localization of FANCF, where it interacts with FANCA, FANCC, and FANCG [30]. In yeast two-hybrid assays a direct interaction between FANCF and FANCG is found through the C-terminal region of FANCF [51,83,93]. The N-terminal part of FANCF is needed to bind the FANCC/FANCE complex, although no direct interaction between FANCF and FANCC, or FANCE alone, has been found. Also this region seems to be necessary for stabilizing the FANCA/FANCG interaction by FANCF [83]. These direct interactions of FANCF with FANCG and FANCC/FANCE and the indirect interaction with FANCA, may implicate FANCF as a key protein in the assembly of a large FA core complex in the nucleus, bringing together the FANCA/FANCG/FANCB/FANCL and the FANCC/FANCE subcomplexes.

#### 4.4. *FANCL*

This protein was identified by protein purification with antibodies against FANCA. FANCL is present both in the cytoplasm and in the nucleus [88,94]. Previously, it was assumed that BRCA1 with its RING finger motif was responsible for the monoubiquitination of FANCD2 [47]. This idea was subsequently disputed because siRNA knock-down of BRCA1 or mutation of the RING finger motifs in BRCA1 did not affect monoubiquitination of FANCD2 [148]. Later, with the finding of FANCL and its RING domain it became evident that FANCL is much more likely to be responsible for monoubiquitination of FANCD2 [94,98]. Although

it is logical to assume that FANCD2 and FANCL at some stage engage in a direct interaction, no direct interaction has been detected so far. It is more likely that FANCE is the missing link between FANCL and FANCD2, bridging the E3 ligase with its substrate. The only direct interaction found for FANCL is the binding with FANCA and FANCB, which seems to be a similar binding as has been observed for FANCE, FANCC, and FANCF. FANCL and FANCB need to bind first before they can interact with FANCA (Medhurst et al., unpublished data).

#### 4.5. FANCM

FANCM, like FANCL and FANCI (see below), also has functional domains: a DEAH-box helicase domain, which seems to render an ATP-dependent DNA translocase activity to FANCM, and an endonuclease domain homologous to ERCC4/XPF. FANCM appears to be the human ortholog of archaeal DNA repair protein Hef, which is probably involved in processing of stalled replication forks [96,102]. FANCM is post-translationally modified in response to DNA damage or replication block, which is not dependent on the ubiquitin ligase activity of the core complex. Furthermore, FANCM becomes hyperphosphorylated after DNA damage, possibly by ATR, and is capable of entering the nucleus on its own. Without FANCM, the initial interaction of FANCA and FANCG seems to be compromised, indicating that FANCM is involved in the initial steps of the FA core complex assembly. Additionally, FANCA and FANCL show deficient nuclear localization in FA-M cells, further suggesting a role for FANCM, not only in the stabilization of the core complex, but also in its nuclear localization [96].

#### 4.6. FAAP100

FAAP100 (Fanconi Anemia Associated Protein of 100 kDa) was identified by protein purification experiments using antibodies against FANCA, and was found simultaneously with FANCL, FANCB and FANCM. FAAP100 thus represents a candidate-new FA gene and complementation group. FAAP100 is not likely to be encoded by the *FANCI* gene, since normal protein expression in FA-I cells was found. So far, no FA patients have been found carrying mutations in FAAP100; until that happens, FAAP100 will retain the status of an 'associated protein' [97].

#### 4.7. FANCI

Although the protein defective in the FA-I complementation group is unknown so far, a clue about its function and position within the FA pathway may be deduced from the characteristics of FA-I cell lines. By focusing on the interactions of the FA proteins within these cell lines, it has become evident that in FA-I cells the core complex is properly formed. This indicates that FANCI, like FANCD2, FANCD1/BRCA2 and FANCI, does not belong to this core complex and must function downstream or independent. However, FANCD2 is not monoubiquitinated in FA-I cells, suggesting that its function is upstream of FANCD2 activation, but downstream of the FA core complex. FANCI may assist FANCL in the activation of FANCD2. The reduced presence of FANCD2-S in nuclear extracts of FA-I cells suggest a function for FANCI in binding FANCD2-S to the chromatin (Fig. 4). As both forms of FANCD2 seem to associate with the chromatin, it is possible that FANCD2 is monoubiquitinated here. However, the faint presence of FANCD2-L in these extracts might point to a downstream function for FANCI, in binding of FANCD2 to the chromatin, but also in the stabilization of FANCD2-L [84].

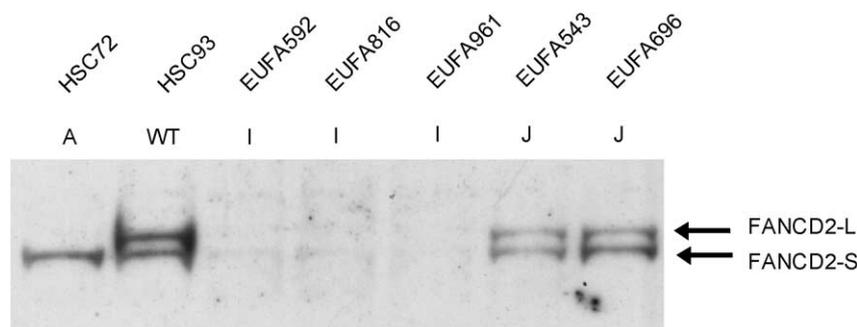


Fig. 4. In FA-J cells, both forms of FANCD2 (FANCD2-S and -L) are clearly detectable in nuclear extracts representing the chromatin fraction. In addition, in FA-I cells, both forms of FANCD2 (FANCD2-S and -L) are hardly detectable in the chromatin fractions of nuclear extracts.

#### 4.8. FANCD2

The FA-D complementation group has been exceptional, as this subset of patients was eventually split up into two separate groups. At first, all complementation data supported the notion that the FA-D group was a single complementation group, composed of 4 unrelated FA patients whose cell lines apparently failed to complement each other [65,136]. In FA-D cells the core complex is intact, indicating that the protein defective in FA-D cells functions downstream of this complex [30,166]. The *FANCD* gene was mapped to 3p22–26 by microcell-mediated chromosome transfer and was finally identified at 3p25.3 [145,162]. However, in two patients no mutations were present, questioning that this was indeed the *FANCD* gene. This situation was resolved by splitting the FA-D group into two separate groups, FA-D1 and FA-D2, of which the latter corresponded to the disease gene at chromosome 3p. The FA-D1 group was found to be defective in *BRCA2* (see below). Unlike most other FA proteins, the *FANCD2* gene product is relatively highly conserved in evolution, with homologs found in *Drosophila*, *C. elegans* and *Arabidopsis thaliana*.

FANCD2 is found in two different states, an inactive state with a molecular weight of 155 kDa (short form, or FANCD2-S) and an active state, with a molecular weight of 162 kDa (long form, or FANCD2-L) [47]. FANCD2 is activated by monoubiquitination on Lysine561. Mutagenesis of this amino acid results in loss of monoubiquitination and failure to complement cross-linker sensitivity of an FA-D2 cell line [47]. This monoubiquitination is dependent upon an intact FA core complex, since in cells that lack the complex ('upstream' complementation groups) only the FANCD2-S form is observed. MMC resistance and FANCD2 activation is restored upon transfection with the corresponding cDNAs. Ubiquitination of FANCD2 occurs during the S phase of the cell cycle. Deubiquitination (by the enzyme USP1) is thought to occur just before mitosis, as cells trapped in this phase do not possess any detectable FANCD2-L [108,141]. The core complex-dependent activation of FANCD2 seems to be a crucial step in the FA pathway, after which FANCD2 co-localizes in subnuclear foci with other proteins including FANCD1/BRCA2, BRCA1, and RAD51 [47,141,158]. The N-terminus of FANCD2, apart from binding FANCE, was shown to bind to the C-terminus of FANCD1/BRCA2. The C-terminus of FANCD1/BRCA2 also binds FANCG [51,60,61,

111,158]. The interaction between BRCA2 and monoubiquitinated FANCD2 seems to be necessary for uploading of FANCD1/BRCA2 into a chromatin complex [158]. Activation of FANCD2 and co-localization with RAD51, BRCA1 and FANCD1/BRCA2 is S-phase specific, suggesting a role for the FA pathway in the repair of DNA interstrand cross-link (ICL) damage during replication, possibly via homologous recombination.

The finding that the DNA damage response protein ATR, which is defective in a Seckel syndrome complementation group, is necessary for FANCD2 monoubiquitination supports this idea [7]. The protein kinase ATR, which is present at the replication fork and becomes activated when damage is encountered. No direct interaction between FANCD2 and ATR has been shown, but ATR mutant cell lines did not show FANCD2 monoubiquitination and siRNA knockdown of ATR showed inhibition of FANCD2 foci formation and induction of chromosomal aberrations. siRNA knockdown of RPA1, a protein that binds ssDNA during DNA replication or replication stress and activates ATR, also showed inhibition of FANCD2 monoubiquitination. These results suggest that RPA1 senses cross-links and through ATR activation is required for FANCD2 monoubiquitination [7].

ATM, defective in ataxia telangiectasia patients, is a protein kinase responsible for NBS1 and BRCA1 phosphorylation [23,48,49]. Disruption of ATM or one of its substrates results in a defect in the IR activated S-phase checkpoint, suggesting that ATM is responsible for a normal S phase checkpoint. FANCD2 is also phosphorylated by ATM at Ser222, which requires the presence of Mre11 and NBS1 [103,142,164]. Phosphorylation of FANCD2 is independent of monoubiquitination, as it still occurs in FA-G cells, and independent of MMC-induced damage, however, this step is dependent on IR damage [103,142]. This is underscored by observations indicating that FA-D2 cells are more sensitive to IR, a feature not seen in other FA complementation groups, and exhibit radioresistant DNA synthesis (RDS), a hallmark for a defective S phase checkpoint [33,110]. NBS1 binds directly to FANCD2 and somehow seems to promote phosphorylation of FANCD2 [103]. These data suggest that FANCD2 has a broader function than only in the FA pathway. A second function for FANCD2 in an IR-inducible activation of an S-phase checkpoint seems to be possible.

## 5. Proteins acting downstream of FANCD2 activation

### 5.1. FANCD1/BRCA2

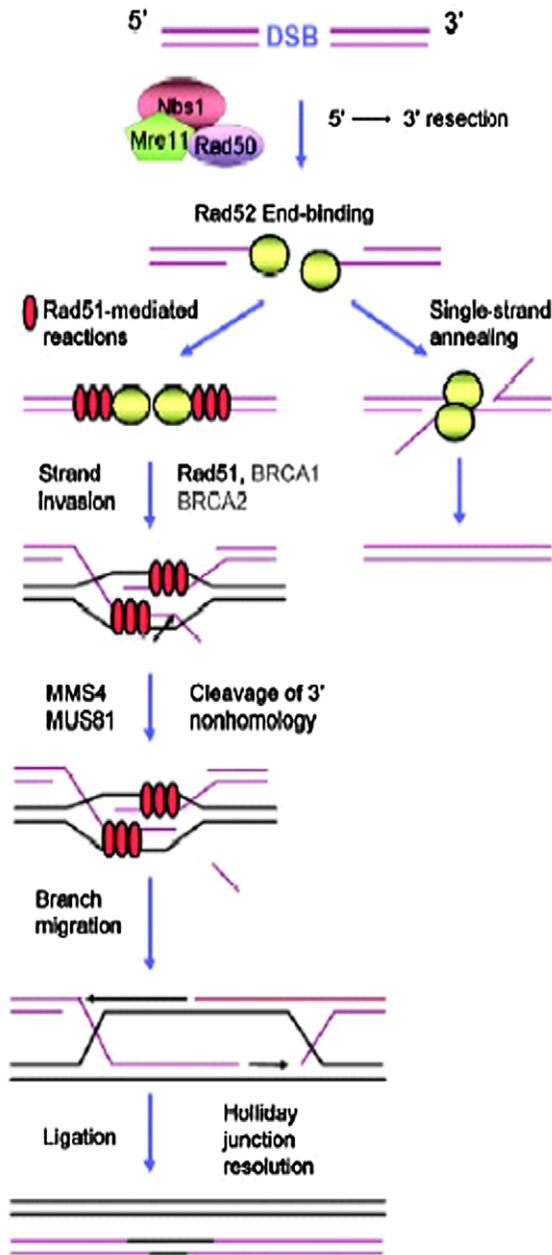
The *FANCD1* gene was identified after screening FA-D1 patients for mutations in *BRCA2* [56]. In multiple FA-D1 patients bi-allelic mutations were found, thereby identifying the *BRCA2* gene as *FANCD1*. FANCD1/BRCA2 functions downstream in the FA pathway, but its main function seems to involve homologous recombination (HR) [30,133,149]. BRCA2 has eight BRC motifs located in the central part of the protein and at least six of these motifs are thought to be involved in direct binding of RAD51, a central HR protein [130,149,163]. An additional tool in the classification of FA patients is RAD51 foci formation, which is observed in all FA complementation groups, except for the FA-D1 group [50].

HR-mediated repair of a double-stranded break (DSB) begins with resection of the DNA strands to yield 3'-overhanging single-stranded DNA (ssDNA) by the MRN complex, which consists of MRE11, NBS1, and RAD50 (Fig. 5) [125]. These ssDNA overhangs are protected by the ssDNA-binding protein RPA. RPA must be displaced for HR to proceed; how this is achieved is not known. *In vitro*, RAD52 can help in this dislodgement, while the C-terminus of FANCD1/BRCA2 seems to be involved in this process as well, through its oligonucleotide/oligosaccharide binding folds (OBs). FANCD1/BRCA2 is able to bind to ssDNA through these domains, which are also found in RPA. RAD51 coats single-stranded DNA substrates, to form a helical nucleoprotein filament that is essential for recombination reactions. RAD51-coated ssDNA initiates strand invasion and ultimately allows recombination between the DNA strands to occur [132,149]. RAD51 filament formation on ssDNA is presumably directed through the interaction with the central BRC repeats of FANCD1/BRCA2, since introduction of peptides resembling BRC repeats effectively blocks RAD51 nucleoprotein filament formation. Similarly, established RAD51 nucleoprotein filaments are dissolved by these BRC repeat peptides, suggesting a role for FANCD1/BRCA2 in removal of RAD51 in later stages of HR [25]. MUS81/MMS4 subcomplex, which possesses endonuclease activity, is involved in the resolution of Holliday junctions, but is also thought to resolve the intermediates arising after stalled replication forks have regressed into a "chicken foot" structure [125].

The FANCD1/BRCA2 protein was found to directly bind to the FA proteins FANCG, FANCD2, and to co-immunoprecipitate with FANCE [60,61,158]. The C-terminal part of FANCD1/BRCA2 interacts with FANCD2, which seems to be dependent on the monoubiquitinated form of FANCD2, since in FA-G cells, which lack FANCD2-L, this interaction does not occur [60,158]. Furthermore, monoubiquitinated FANCD2 is necessary for loading of FANCD1/BRCA2 onto damaged chromatin [158]. FANCD2 also directly interacts with FANCE, suggesting that co-IP of FANCE and FANCD1/BRCA2 is based on an indirect interaction. However, in FA-D2 cells, which lack the FANCD2 and FANCD1/BRCA2 interaction, FANCE and FANCD1/BRCA2 could still be co-immunoprecipitated [158]. The precise implications of this interaction are not known. The FANCG interaction is not confined to a single site; instead, FANCG is able to bind both the N- and the C-terminal regions of FANCD1/BRCA2, on either side of the central BRC domains. The TPR motifs found in FANCG, which are involved in protein-protein interactions, seem ideally suited to accept alpha-helices from target proteins. In the C-terminal tertiary structure of FANCD1/BRCA2, 10 of these alpha-helices have been identified and this domain is involved in binding of FANCG, possibly through the TPR motifs [16,61]. Although FANCG and RAD51 do not bind each other directly in a yeast two-hybrid assay, they do seem to co-localize to damage-inducible foci, suggesting an indirect interaction between the two, mediated by FANCD1/BRCA2 [61].

These interactions can be combined into a model for HR, in which signals triggered by DNA damage induce FANCD1/BRCA2-dependent translocation of RAD51 to repair sites [149]. First, FANCD1/BRCA2 binds RAD51 in an inactive state, possibly in a structure recently proposed, in which the BRC repeats wrap around the RAD51 ring structure. This brings the N- and C-terminal regions of FANCD1/BRCA2 in close proximity of one another [131]. Since FANCG binds both the N- and C-terminal regions of FANCD1/BRCA2, it is possible that FANCG binds both these regions of FANCD1/BRCA2 simultaneously, thus stabilizing the FANCD1/BRCA2/RAD51 structure until the correct signal is received for unloading of RAD51 onto ssDNA to form the DNA-protein filament structure that is essential for HR to take place [60,61]. FANCD2 possibly directs and uploads this RAD51/FANCD1/BRCA2/FANCG complex onto the chromatin at the damaged site [158]. RPA is then re-

### Homologous Recombination (HR)



### Nonhomologous end-joining (NHEJ)

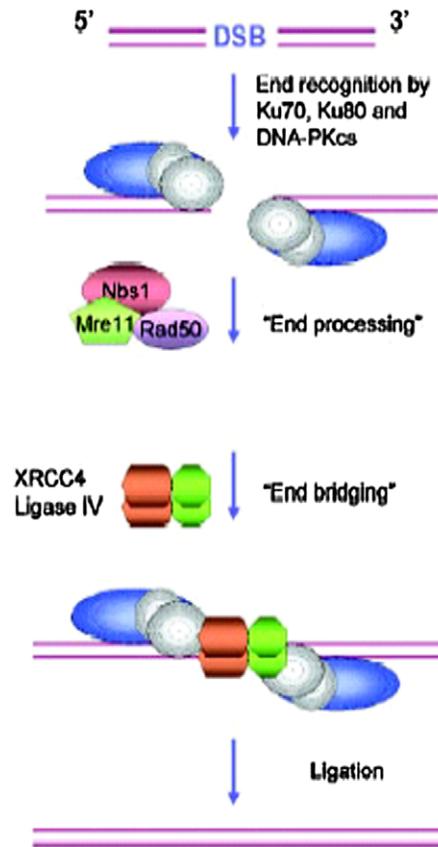


Fig. 5. Schematic representation the homologous recombination (HR), single-stand annealing (SSA) and non-homologous end joining (NHEJ) repair pathways (Figure reprinted, with permission, from the *Annual Review of Biochemistry*, Volume 73 © 2004 (Sancar et al.) [125] by Annual Reviews www.annualreviews.org).

placed from the ssDNA, possibly by the OB domains of FANCD1/BRCA2. Next, a signal is given for the unloading of RAD51. FANCD2 could be part of this signal by replacing FANCG at the same C-terminal region

of FANCD1/BRCA2 and thus promote RAD51 unloading onto ssDNA [60]. FANCD1/BRCA2 then regulates RAD51 nucleoprotein filament assembly onto ssDNA, which is needed to initiate strand invasion

and repair of the lesion [132,150,167]. After the DNA damage has been repaired and recombination completed, the DNA damage signals are eliminated, and FANCD1/BRCA2 is thought to disrupt the RAD51 filaments and remove them from the DNA.

## 5.2. FANCI/BRIP1

FANCI/BRIP1 is one of the first FA proteins that clearly associates with DNA, through its DEAH-box helicase domain [19,85]. FANCI is an ATP-dependent helicase, like the XPD protein in xeroderma pigmentosum, and functions by binding and then unwinding DNA/DNA and DNA/RNA substrates in a 5' → 3' direction [20]. FANCI is a member of the family of RecQ DEAH helicases, which are very efficient in unwinding non-Watson–Crick DNA structures, such as Holliday junctions that arise during HR and during the repair of stalled replication forks [120]. It is thought that these helicases play indeed an important role in the repair of stalled replication forks. The unwinding of DNA helices in a stalled replication fork by FANCI would allow the DNA repair proteins, for example HR or NER proteins, to access the specific damaged site and facilitate DNA repair of an ICL. FANCI preferentially unwinds a forked duplex substrate with a 5'-ssDNA tail and 5'-flap substrates and is able to release the third strand of the homologous recombination intermediate D-loop structure, irrespective of tail status [53]. FANCI also contains a C-terminal BRCA1-binding domain, again linking the FA pathway to the HR repair pathway. This binding is dependent upon the phosphorylation of FANCI at Ser990 during S and G2/M phase and seems to be necessary for a normal G2/M checkpoint function [170]. However, recently it was shown that DT40 cells lacking FANCI do not show defective homologous recombination or a defective G2/M damage checkpoint [17].

Since in both the FA-J and FA-D1 groups the FA core complex is still formed and FANCD1/BRCA2 is an important member of the homologous recombination pathway, it may be argued that FANCI and FANCD1/BRCA2 are not genuine FA genes, but merely associate with the FA pathway. However, the clinical characteristics of FA-J patients, in contrast to the FA-D1 patients, are not different from other FA patients, while FANCI has not been associated with any other pathway so far. This would suggest that FANCI is a true FA protein, with a function downstream in the FA pathway.

## 6. The FA pathway: Model and function

### 6.1. FA core complex assembly

Based on available data, a model for the FA pathway can be proposed. It has become clear that all FA proteins interact with each other, directly or indirectly. Thus, the known interactions can be fitted into the following hypothetical model (Fig. 6).

In this model, the FA proteins mainly function in the nucleus, but the first steps towards a communal function already take place in the cytoplasm. The proteins detected in the cytoplasm are FANCA, FANCB, FANCC, FANCG and FANCL. FANCA and FANCG interact directly in the cytoplasm of the cell and move into the nucleus by active transport, since this complex is too large to allow passive transport. For active transport a NLS sequence is needed, which might be provided by both the FANCB and FANCA proteins, since FANCB together with FANCL binds to FANCA [7] (Medhurst et al., unpublished data). Perhaps, FANCG retains FANCA in the cytoplasm through binding to its NLS sequence and, once FANCB, and FANCL have bound, a conformational change takes place, upon which FANCG exposes the NLS sequence, where after the complex can be imported into the nucleus. This is supported by the observation that without FANCB (in FA-B cells), FANCA is mainly found in the cytoplasm [30]. Alternatively, both subcomplexes of FANCA/FANCG and FANCB/FANCL could move separately into the nucleus through their NLS sequences and form a larger complex there. This interaction into a larger subcomplex may be required to maintain nuclear localization.

In the nucleus, FANCM is thought to bind to and move along the DNA in search of the lesion. Since FANCM is necessary for the FANCA–FANCG interaction and for nuclear accumulation of FANCA as well, it seems logical that FANCM binds both FANCA and FANCG. However, the finding that FANCM is found exclusively in the nucleus, while FANCA is found both in the nucleus and the cytoplasm, would be in conflict with this idea [96]. It is more likely that FANCM initiates the cytoplasmic subcomplex of FANCA and FANCG by, for example, a signalling protein, which would explain why this interaction is compromised in FA-M cells. Next, in the cytoplasm or in the nucleus, the FANCB/FANCL subcomplex joins the FANCA/FANCG subcomplex. At the same time, FANCC enters the nucleus on its own, or associated with a carrier protein, and is bound there

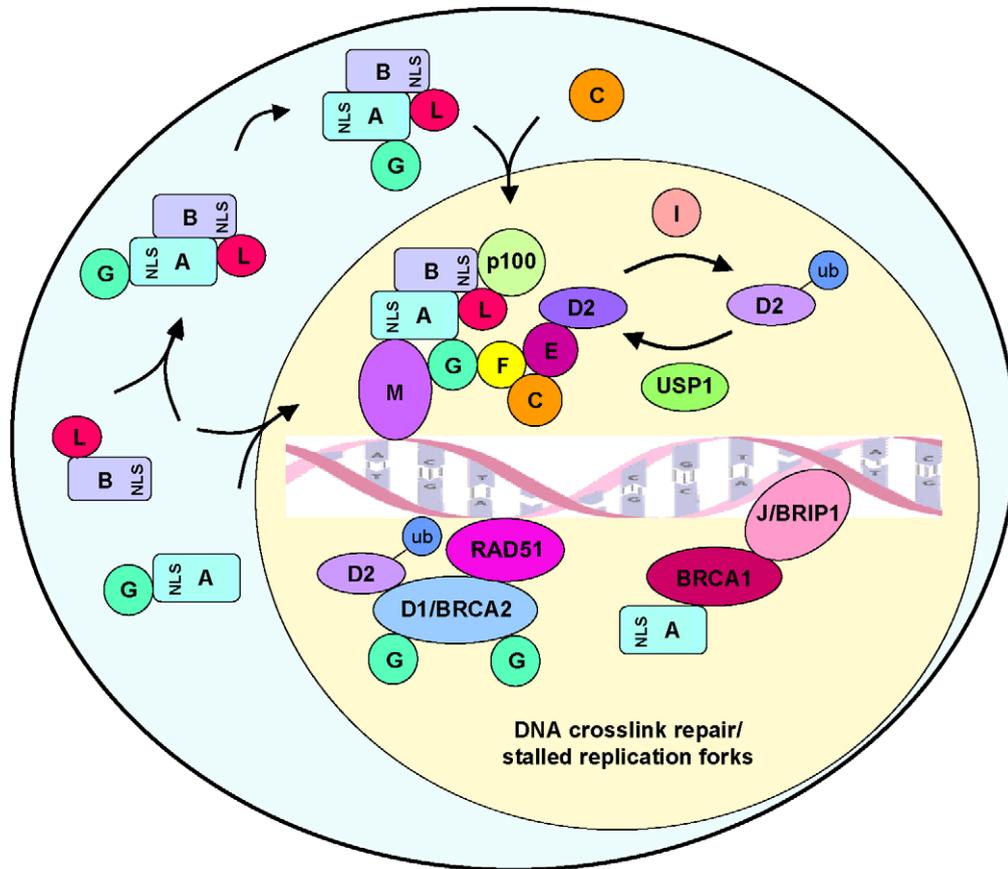


Fig. 6. Current view of the FA pathway showing how the FA proteins interconnect. FANCA and FANCG bind in the cytoplasm and presumably interact with FANCB and FANCL in the cytoplasm or in the nucleus. In the nucleus it combines with FANCM. Binding of FANCF, -E, and -C is essential to stabilize the interaction between FANCM and the FANCA/B/G/L sub-complex and to form the core complex. FANCD2 interacts with this complex through FANCE. The E3-ubiquitin ligase FANCL activates FANCD2 by monoubiquitination at Lys561. FANCI is also required for FANCD2 activation, but its precise role remains to be determined. De-activation is thought to occur through USP1, a de-ubiquitinating enzyme. Activated FANCD2 then co-localizes with BRCA2/FANCD1, RAD51, and BRCA1 in nuclear foci that form at DNA damage sites. FANCD2 is also thought to be involved in uploading of BRCA2 onto the chromatin. FANCD2 binds directly to FANCD1/BRCA2, an interaction that depends on monoubiquitination of FANCD2 and therefore represents a late event in the pathway. The intimate connections between FA and HR proteins at the chromatin level may facilitate repair of cross-link damage and/or resolution of stalled replication forks. Figure reproduced, with permission, from the thesis of M. Levitus.

by FANCE. Next, this subcomplex of FANCC and FANCE associates with FANCF, which was already bound to the complex via FANCG. This makes FANCF the bridge between on one side FANCM, FANCA, FANCG, FANCB, and FANCL, and on the other side FANCC and FANCE.

The binding of FANCF, FANCE, and FANCC is thought to be necessary for stabilizing the interaction of FANCM and the FANCA/-G/-B/-L subcomplex. This major complex of FA proteins is called the core complex. Since a defect in every member results in a lack of FANCD2 ubiquitination, the entire core complex is thought to be responsible for FANCD2 activa-

tion by monoubiquitination. Most likely, FANCE binds FANCD2 and becomes the bridge between the E3 ligase FANCL and its substrate, FANCD2. FANCI may assist FANCL by binding or locating FANCD2-S to the chromatin and allow an interaction with the core complex. After FANCD2 is monoubiquitinated, the protein forms subnuclear foci with BRCA1 and RAD51 and binds directly to FANCD1/BRCA2, an interaction that depends on monoubiquitination of FANCD2 and therefore represents a late event in the pathway.

The interaction seen with FANCD1/BRCA2 might be one of the most relevant for the FA pathway, since FANCD1/BRCA2 is a key protein in the HR

pathway. A second interaction and link with the HR pathway is through FANCI, which binds BRCA1 and could participate in HR repair by unwinding DNA in 5' → 3' direction through the DEAH helicase domain. FANCD1/BRCA2 binds directly to RAD51, and FANCG, and possibly "indirectly" to FANCI, perhaps via FANCD2. This could lead to a model in which monoubiquitinated FANCD2 recruits FANCD1/BRCA2 to damaged sites in the chromatin [158]. Meanwhile, FANCG stabilizes the complex of FANCD2, FANCD1/BRCA2, and RAD51, until the damaged site is reached, suggested by the large overlapping binding sites of FANCG with FANCD1/BRCA2. Then, after FANCD2 may have replaced FANCG; FANCD1/BRCA2 can load RAD51 onto ssDNA to initiate homologous recombination. This would suggest that the FA pathway functions to assist FANCD1/BRCA2 in HR.

FANCD2 seems to have a dual role, one specific for the FA pathway in resolving cross-links and a second function in activating an S-phase specific checkpoint through phosphorylation by ATM after IR-induced damage. This phosphorylation requires MRE11 and NBS1, to which FANCD2 binds directly, but is independent of its interaction with FANCD1/BRCA2 [157].

It is possible, though, that the interactions of the different FA proteins with the HR proteins occur independently of the general FA pathway that resolves cross-links. These interactions could be part of a second independent function of these FA proteins, as is observed for FANCD2. The FANCC protein has been reported to interact with a number of other proteins, such as cdc2, Hsp70, and p53, and also might have a separate function in cell cycle control, hematopoiesis, apoptosis or tumorigenesis. The interaction seen between FANCA and BRCA1, would sustain both hypotheses as BRCA1 is also involved in numerous processes, one of them is HR and DNA repair, but FANCA might also have an additional function via BRCA1 in one of the other processes, such as a cell cycle checkpoint control. If this is true and the FA proteins have additional tasks, then one would suspect to see more severe phenotypes among patients of certain complementation groups, for example, FA-C patients. This is not the case. Even more peculiar is that certain mutations in the FA-A and FA-C group cause a mild phenotype, thereby contradicting the notion of separate functions, independent of the FA pathway.

## 6.2. A function for the Fanconi anemia complex?

The actual function of the FA pathway is still unknown. Apart from several theories and speculations no evidence has been shown yet to convincingly support a function for the pathway. The recently discovered FANCM and FANCI proteins with their DNA binding and modifying properties are finally hinting toward a function and support the general theory that the FA pathway is in one way or another involved in DNA repair. It seems likely that the FA proteins are involved in HR repair, since a number of FA proteins interact with the BRCA pathway, or – in the case of FANCD1 – belong to this pathway. Another clue comes from the chromosomal breakage and the late S-phase cell cycle arrest seen in FA cells. Both become apparent only after FA cells have undergone DNA replication. Furthermore, the type of damage that FA cells are sensitive to, DNA interstrand cross-links (ICLs), are usually encountered during replication in S-phase. Activation of FANCD2 through monoubiquitination is also carried out in S-phase and is needed for the chromatin uploading of BRCA2, whereas de-ubiquitination occurs before M phase. Finally, co-localization of FANCD2 with RAD51 and BRCA1 in nuclear foci is also S-phase specific. All these observations lead to a general idea that FA proteins are involved in the repair of ICLs by the HR pathway after the lesions have been encountered during replication [2,108,125,141].

DNA lesions induced by monofunctional alkylating compounds are efficiently repaired by nucleotide excision repair pathway (NER); this is not as straightforward for ICLs, since they involve both strands of the DNA helix. Therefore ICLs are usually detected during replication, when the ICL connects both DNA strands so that they can no longer be separated. The replication fork cannot proceed and is therefore stalled due to the ICL [125].

ICLs are highly toxic lesions and repair of ICLs is thought to involve multiple pathways (Fig. 7,1–7,2) [32]. Basically, the repair process involves the sensing and the excision of the ICL. In mammalian cells subsequent repair of the lesion is thought to proceed through formation of a DSB and repair via homologous recombination [14,105,106]. A hypothetical model for the role of the FA proteins in ICL repair can be proposed. Initially the cross-link has to be sensed during replication, which might be done through hMutSβ or ATR, a sensor in replication stress [125,171]. Like in bacteria, the stalled fork could regress and form a chickenfoot structure, however, the

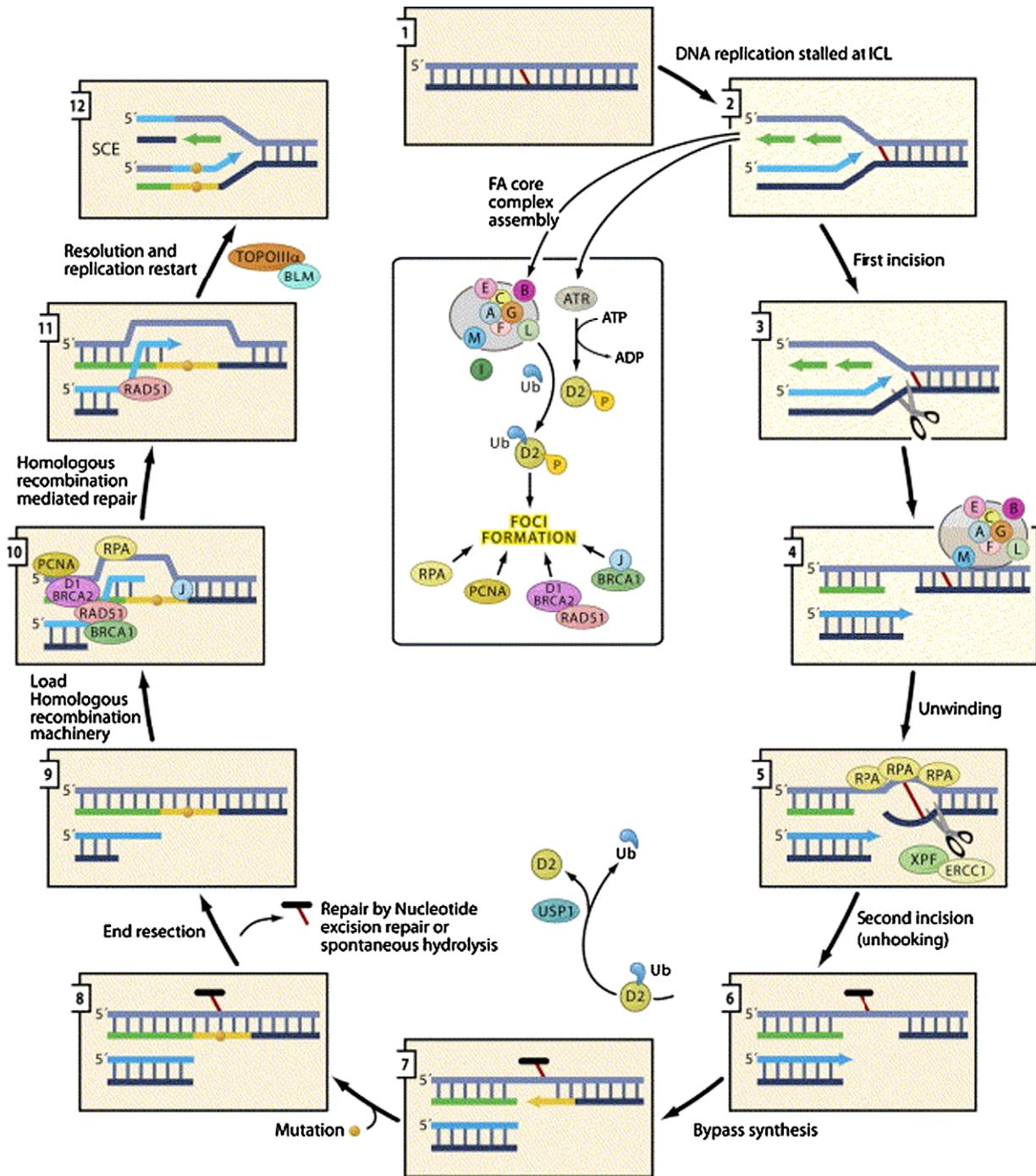


Fig. 7. Resolution of a DNA cross-link during DNA replication. When a DNA cross-link is encountered during replication, the replication fork will stall (1–2). One of the DNA strands is cut, which results in a double stranded break (DSB) and a strand with a ssDNA gap. This DSB can be visualized by the  $\gamma$ -H2AX protein that is shown to bind to these lesions. To resolve the cross-link, ERCC1 and XPF make another incision opposite of the ssDNA gap. This allows the cross-link to be removed from one strand. The ssDNA gap can now be fixed by translesion synthesis (TLS) over the damaged site at the complementary strand (3–7). Excision repair and subsequent normal DNA synthesis ensures repair of the complementary DNA strand (8–9). Homologous repair is needed to repair the DSB and to re-initiate replication (10–12). (Figure reproduced with permission from *Cell* (Niedernhofer et al.) [106]. © 2005, Elsevier.)

mechanism by which the replication fork is able to regress is currently unknown. Next, the stalled fork is cut, perhaps by MUS81/MMS4, which eliminates the fork structure and thereby transforms the stalled replication fork into a collapsed fork [115,128,132] (Fig. 7,3–7,4). A collapsed fork consists of two DNA helices, one ending in a DSB and the other having an ssDNA break. These ssDNA ends can bind RPA to which ATR can bind; both are necessary for FANCD2 activation. ATR also is involved, by providing an S-phase delay through the inhibition of replication origin firing. FANCM, which is already post-translationally modified, contains several ATR phosphorylation sites and becomes hyperphosphorylated after DNA damage. FANCM might therefore serve as a substrate through which ATR regulates FANCD2 monoubiquitination. FANCM is also needed for core-complex assembly and is thought to translocate along the DNA through the DEAH-box helicase domain. This could imply that FANCM assembles the core complex and subsequently translocates it along the DNA as a ‘monitor’ to sense and locate the sites of DNA damage [7,96,125,147]. Now that the core-complex has been assembled, FANCD2 which is located to the chromatin through FANCI can bind FANCE and can become activated by FANCL. This activated FANCD2 at the chromatin then promotes activation of multiple DNA repair pathways, such as translesion synthesis (TLS) and HR. This is supported by the finding of genetic epistasis between the FA pathway and TLS, and the HR pathways [107].

Simultaneously, the ICL is resolved by a second incision made on the other side by members of the nucleotide excision repair (NER) pathway (ERCC1/XPF), which is a 5′-endonuclease, thereby releasing the ICL from one DNA strand (Fig. 7,5) [27,105,125]. ERCC1/XPF only functions once an adjacent 3′-ssDNA region is formed [11,26]. In NER, this ssDNA region is formed by XPG, a 3′-endonuclease, in a stalled replication fork MUS81/MMS4 provided this ssDNA region by collapsing the fork (Fig. 7,3) [54,105,125]. The BLM protein, which is a 3′ → 5′ DNA helicase, found in complex with the FA core complex proteins, could allow ERCC1/XPF to access the DNA [88]. It is possible that  $\alpha$ SpII $\Sigma^*$ , which is found in a complex with both FANCA and XPF, promotes XPF relocalization and incision [135,144]. It is, however, unlikely that the core-complex is involved in the recruitment of XPF, since FA cells are apparently not deficient in the incision of ICLs [123]. ERCC1/XPF is also reported to function independently of replication during the G1 phase [123]. If this is so, the ICL can

only be incised by ERCC1/XPF if a 3′-ssDNA nick has occurred, since ERCC1/XPF only functions when an adjacent 3′-ssDNA region is present [11,26]. However, some investigators do report dual incision activity of ERCC1/XPF on both sides of the lesion [80,171]. Processing of the unhooked ICL then occurs during S-phase, as described above, when the replication fork becomes stalled at the unhooked ICL and a DSB is formed.

The ssDNA gap now formed in the complementary strand after ERCC1/XPF has released the ICL is filled by the TLS pathway. Now the ICL can be excised, perhaps via the complete NER pathway to remove the ICL and the resulting ssDNA gap is filled by the normal DNA synthesis machinery (Fig. 7,6–7,9). HR then initiated, first the DSB ends are resected by the MRN complex, after which uploading of FANCD1/BRCA2 onto the chromatin through FANCD2 initiates strand invasion by RAD51. The now emerging homologous recombination intermediate D-loop structure forms a substrate for FANCI, a 5′ → 3′-DEAH-box helicase. FANCI is shown to unwind the invading third strand of a D-loop irrespective of tail status. However, FANCI seems to be unable to unwind artificial Holliday junctions, contradicting a role in Holliday junction branch fork migration, as proposed for other RecQ helicases. Holliday junctions may be resolved by MUS81/MMS4, perhaps assisted by BLM protein, although there is no evidence to support this idea. Finally, replication can restart (Fig. 7,10–7,12) [105].

In this model regression of the stalled replication fork creates two ssDNA ends similar to DSB ends. These ssDNA ends activate the FA pathway as described above. One of the FA proteins is thought to possess 3′ → 5′ exonuclease activity, if applied to the two ssDNA ends of the regressed fork, it would create a 5′-ssDNA tail or overhang (Fig. 7B\*). This 5′-overhang in a duplex structure forms the perfect substrate for another downstream component FANCI, a 5′ → 3′ DEAH-box helicase. FANCI then unwinds the forked duplex substrate in a 5′ → 3′ direction, which would allow other proteins to access the DNA in the stalled replication fork, such as MUS81/MMS4 [53]. However, since FA cells are not deficient in the incision of ICLs and DSB formation, this implies that the FA pathway functions downstream of both DSB formation and incision. These results contradict the supposed function of FANCI in assisting MUS81/MMS4 in the formation of a DSB and indirectly also in the ability of ERCC1/XPF to incise on the 5′-side of the lesion [123].

DSB repair in yeast and *E. coli* is mainly performed by HR, while in mammalian cells three DSB repair systems are present: HR, non-homologous end-joining (NHEJ), and single-strand annealing (SSA). It was previously thought that NHEJ is the most important for DSB repair, and HR was mainly used during meiosis, but now it seems that HR and NHEJ both play important roles in general DSB repair (Fig. 5) [12,13,43,63,70,72,115,117].

DSB formation and repair during replication is somehow different from the general variant, since with these DSBs only one end is available to start repair from, whereas in the general form, both ends of the DSB are available for repair. DSBs that are present in collapsed replication forks are thought to be resolved by post-replication repair, which is made up of three different mechanisms, HR, TLS and NHEJ. HR and TLS seem to be the most important, although NHEJ is also thought to be involved, which seems to fit with the model described above. Both can be subdivided into error-free and error-prone repair. HR is an error-free pathway that uses identical sister chromatids for repair, while the error-prone pathway of TLS merely bypasses the lesion and continues replication; NHEJ just pastes both strands together irrespective of their sequence homology [8,32,56,82,92,100,116].

In *S. cerevisiae*, post replication repair (PRR) is organized by the RAD6 epistasis group, which includes RAD18. RAD6 itself encodes an E2 ubiquitin ligase, while RAD18 is an E3 ubiquitin ligase that delivers RAD6 to the damaged site. Hence, in this system ubiquitin serves as a signal that switches replication into PRR at sites of replication block [56]. All observations so far made for the FA proteins point to a function in the resolution of stalled replication forks after cross-link damage, which was first suggested by Thompson et al. [144]. This is supported by the observation that FA cells show predominantly deletion mutations, which occur as a result of collapsed replication forks, and the observation that activation of the FA pathway occurs during replication in S-phase [54,81,113]. However, it seems unlikely that FA proteins are directly involved in HR or TLS, since FA cells are not particularly sensitive to UV or IR. Furthermore, no direct role in these repair pathways has been found, merely a promoting or assisting role for the FA proteins in HR repair and a minor role in SSA via BRCA1 [104,109]. Additionally, FA cells seem to have no problems converting a cross-link into a DSB; however these DSB persist for a long time, indicating that the FA proteins are involved in the processing and

repair of these lesions [123]. Finally, components of both HR and TLS are conserved from bacteria to humans during evolution, while the FA proteins are only found in vertebrates, with the exception of FANCD2, FANCL, FANCM, and FANCI.

Overall, these data would suggest an assisting or perhaps coordinating role for the FA proteins in repair of ICL-specific replication repair, similar to the RAD18 protein. The FA proteins are involved in the monoubiquitination of FANCD2, so perhaps the FA pathway and FANCD2 act as Rad18 in stabilizing the stalled replication fork, while they coordinate and recruit the different repair pathways during the induced S-phase delay, thereby maintaining replication fork progression (Fig. 5). If FA proteins are involved in stabilizing the stalled replication fork, it would also explain why after HU treatment FANCD2 is activated, while no cross-link damage is present. The fork is stalled and needs to be stabilized by the FA pathway until replication can proceed, but does not require repair. Evidence for a repair-coordinating function of the FA proteins comes from the observation that FA proteins and FANCD2 associate with chromatin during S-phase and provide a direct link with certain repair pathways [99,101,107,114,158]. In addition, FANCA has been found to interact with BRG, a component of the SWI/SNF complex, suggesting a role in chromatin remodeling to allow repair of damaged DNA [110].

HR repair is the most preferred type of repair, since this is error-free, and FA proteins seem to be involved in this type of repair [27,54,125,144]. In the absence of a functional FA pathway, the fork can no longer be effectively repaired. The FA proteins are not available to stabilize and coordinate repair of the replication fork and faulty repair is initiated (Fig. 8). This could be done by NHEJ pathway, since FA cells show increased NHEJ DSB repair infidelity and decreased fidelity of V(D)J joint formation, which is also directed via NHEJ [81,134]. Furthermore, NHEJ functions independently of the FA pathway and is therefore available when HR is not [104]. Initiation of error-prone repair could take place via RPA, which apart from its involvement in replication and HR, is phosphorylated by DNA-PK, a member of the NHEJ pathway [4].

Activation of the error-prone NHEJ pathway has been suggested to be responsible for genomic instability, deletion proneness and even leukemia, traits that are seen in FA patients and their cells [118,134]. However, normal levels of NHEJ have also been reported in FA cells, suggesting that in the absence of the FA proteins an alternative error-prone pathway is used than the NHEJ pathway [104].

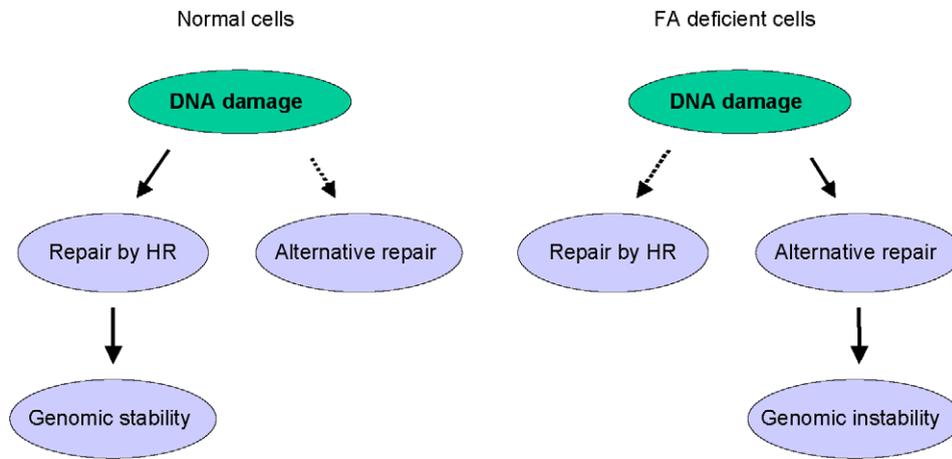


Fig. 8. In normal cells the FA pathway initiates the homologous recombination pathway to ensure correct repair of a DNA cross-link. The alternative, error-prone pathways, only play a minor role. In an FA-deficient cell, homologous recombination can no longer be utilized; therefore the alternative, error-prone pathways, such as NHEJ, come into action. This situation leads to genomic instability and cell death.

FANCD2 is the odd protein amidst the FA proteins since it seems to play a dual role, one specific for the FA pathway in resolving cross-link damage and another role in general DSB repair via ATM after IR damage, which activates an S-phase-specific checkpoint [157].

### 7. Is the FA pathway involved in the origin of sporadic cancer?

All cells in the human body are constantly dealing with DNA damage generated by exogenous sources, such as UV light, tobacco smoke, or a combination of environmental agents. Endogenous factors, generated inside the cells, are also responsible for mutagenesis; such factors include reactive oxygen species, alkyl groups and DNA mismatches due to inaccurate DNA replication. Since DNA replication is so incredibly accurate, it seems that the DNA repair systems available are highly competent in dealing with most insults, resulting in a low spontaneous mutation rate. Mutations can only persist in cells if checkpoints allow them to continue to proliferate. Cancer is an accumulation of multiple mutations within a single cell that allows this cell to break free of its (growth) limitation and eventually become a tumor. Generally, it is believed that the normal or spontaneous mutation rate cannot account for the large number of mutations present in cancer cells. Therefore, it is thought that a cell must first acquire a mutator phenotype, a phenotype that allows for an increased mutation rate, to accumulate the large number of mutations observed. This phenotype

is likely to occur, when a gene involved in maintaining normal genetic stability is mutated or inactivated. This will render the cell genetically unstable, so that mutations can accumulate in oncogenes and tumor suppressor genes or other genetic stability genes, thereby further increasing the mutation rate, ultimately leading to cancer [90,91]. The FA genes are involved in maintaining genomic stability and inactivating one of them will lead to a mutator phenotype, which is suggested to be an early event in tumor formation.

The *FANCB* gene is the first gene that is located on the X chromosome, Xp22.31. Therefore, *FANCB* is the only FA gene that has only one functional gene copy present in the cell; in males only one X chromosome is present and in females one X chromosome is subjected to X inactivation. This situation implies that only one *FANCB* gene copy needs to be inactivated in order to obtain a FA cell, with all its characteristics. This does suggest that in female mutation carriers due to random X inactivation 50% of their cells should be FA cells, which implies that in contrast to other carriers of a FA mutation, these females might well have an increased risk of developing those tumors, for which FA patients are highly susceptible. In mosaic FA patients, a proliferative advantage of healthy blood cells has been observed, so that these patients show no hematological problems [52,155]. A similar mechanism is observed in female *FANCB* mutation carriers. Both, in the blood and fibroblasts almost completely skewed X inactivation was observed against the chromosome having the mutated allele; therefore, no immediate hematological problems are expected in these females. However, a small proportion of the other cell types, such as fibro-

lasts could express the mutant *FANCB* allele. As yet, only three female carriers have been studied, so that the possible risk for development of squamous cell carcinomas and other FA-related tumors cannot be excluded at present in these women.

Other FA proteins and genes may be important for sporadic tumor formation. *FANCF*, which is located in a hot-spot region for hypermethylation, was shown to be silenced in a sporadic AML cell line through methylation of its promoter sequence. However, no further examples of *FANCF* methylation have so far been detected in sporadic cases of AML. Interestingly, *FANCF* silencing by methylation has also been observed in a subset of ovarian tumor cell lines and in 20% of primary tumors, indicating this was not just a random effect seen in a single AML cell line. These cell lines and tumors have a defective FA pathway and therefore should exhibit cisplatin hypersensitivity. Demethylation of *FANCF* restored *FANCF* mRNA expression and the FA pathway and restored cisplatin resistance. This phenomenon of phenotypic reversion was also seen in some tumor cells [143,146]. These findings have led to a model for tumorigenesis based on *FANCF* silencing. A cell gains genomic instability through silencing of *FANCF* and by acquiring multiple mutations develops into a tumor cell. This tumor is highly sensitive to cisplatin and can be treated accordingly. However, after initial treatment with cisplatin, some cisplatin-resistant cells may arise, due to demethylation of *FANCF*. Treatment of these tumors with an FA pathway inhibitor will sensitize these tumor cells to cisplatin once again and might prove to be an effective means to treat sporadic cancer [143].

Since only one *FANCB* allele is active or expressed in all human tissues, a single mutation in this gene would render it inactive and create a (genomically unstable) FA cell. The average spontaneous mutation rate is in the order of  $10^{-6}$  per gene per cell generation. For genes located on autosomes, when both alleles are present, this would imply a chance of  $10^{-12}$  to inactivate a gene by spontaneous mutagenesis [160]. *FANCB* has only one allele, implying that the chance for this gene to become inactivated through spontaneous mutagenesis is  $10^6$  fold higher than for an autosomal stability gene. Since FA cells are prone to undergo apoptosis, only a small proportion of the *FANCB* defective cells would survive long enough to gain multiple mutations in other genes, for example apoptotic genes, and eventually become malignant. Nevertheless, the heightened chance of inactivation would imply that *FANCB* is a vulnerable component in the cell machinery to avoid

genomic instability and ultimately cancer. Overall, the same arguments applied to silencing of *FANCF*, can be applied to *FANCB*, in that a defect in *FANCB* could well be the underlying defective component in a subset of sporadic tumors in the general population. Further investigation into this possibility is needed to substantiate this hypothesis. If indeed the hypothesis were strengthened by additional data, and *FANCB* were still defective in these tumors, these will be hypersensitive to cisplatin and should be treated accordingly.

The proportion of sporadic cancers that are cisplatin-sensitive is relatively small. This may be explained by the apoptotic phenotype of FA cells, since only a small proportion of cells will gain sufficient mutations to become malignant and secondly, this might be due to spontaneous reversion of malignant cells, which would lead to escape from the poor growth ability associated with an FA phenotype. After the initial growth-promoting mutator phenotype, a tumor cell will benefit from obtaining a full growth potential by shedding the now growth-limiting FA phenotype. Treatment of such reverted tumors with cisplatin will only be effective if it were possible to reintroduce the FA phenotype, e.g., by inhibition of the FA pathway.

## 8. Summary and conclusions

Now that most of the FA genes and their proteins have been identified, insight into the specific function of the FA pathway in DNA cross-link repair is emerging. Circumstantial evidence points towards a role of the FA pathway in the repair of stalled or collapsed DNA replication forks. Most of the FA proteins interact to form the FA core complex, whose main function seems to be the activation of FANCD2 by monoubiquitination. However, core complex member FANCM and the downstream proteins FANCI and FANCD1/BRCA2 seem to have additional functions. FANCM and FANCI are both thought to unwind the DNA, while FANCD1/BRCA2 is the only protein for which a clear function in HR has been described and which is probably needed to reinitiate replication. In the absence of the FA pathway, stabilization of replication forks and recruitment of DNA repair pathways is compromised, likely resulting in misrepair, typically resulting in chromosomal instability seen in FA cells, such as gaps, breaks and interchanges. To find a specific function for the FA pathway and link this to the phenotype seen in patients, it is important to learn more about the already identified components, for example

the identification of the actual substrates for FANCI and FANCD1 and the interacting partners of, among others, FANCD2 outside the FA pathway. Furthermore, identification of new components within the FA pathway, such as *FANCI*, might also shed light on its elusive function. Additionally, further investigation into the involvement of the FA pathway in sporadic cancer, might reveal a subset of tumors that are defective in the FA pathway and therefore might be successfully treated with DNA cross-linking agents.

### Acknowledgements

We thank H. te Riele, G.A. Meijer, K.J. Patel, R. Brakenhoff and P.C. Huijgens for valuable comments on the manuscript. This study was financially supported by the FA Research Fund, Eugene, Oregon, U.S.A.

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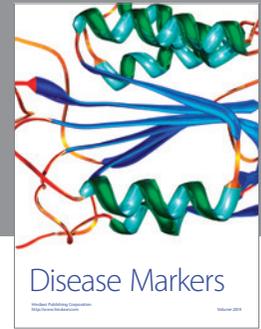
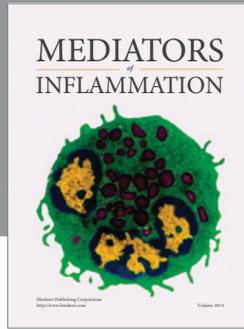
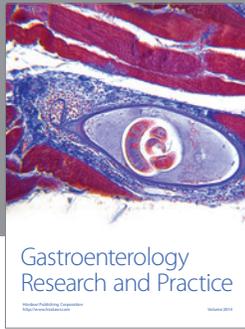
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